INTRAGENOMIC, INTRA- AND INTERSPECIFIC VARIATION IN THE RDNA ITS OF PORIFERA REVEALED BY PCR-SINGLE-STRAND CONFORMATION POLYMORPHISM (PCR-SSCP)

GISÈLE LÔBO-HAJDU*, ANA CAROLINA R. GUIMARÃES*,
ADRIANA SALGADO*, FLÁVIA R. M. LAMARÃO*,
THOMÁZ VIEIRALVES*, JOSÉ JOÃO MANSURE** &
RODOLPHO M. ALBANO***

*Depto. de Biologia Celular e Genética, IBRAG, Universidade do Estado do Rio de Janeiro (UERJ), Rua São Francisco Xavier, 524 - PHLC, sala 205, Maracanã, 20550-013, Rio de Janeiro, RJ, Brasil
**Instituto Metodista Bennett, Rio de Janeiro, RJ, Brasil
***Depto. de Bioquímica, IBRAG, UERJ, Rio de Janeiro, RJ, Brasil
E-mail: lobohajdu@alternex.com.br

ABSTRACT

We have applied the polymerase chain reaction-based single-strand conformation polymorphism method (PCR-SSCP) to detect sequence variation in the first and second internal transcribed spacers (ITS1, ITS2) of ribosomal DNA (rDNA). These polymorphic DNA sequences have been used as additional characters to separate species of *Cliona* Grant, 1826 (Hadromerida) and to estimate levels of variability within and among populations of *Hymeniacidon heliophila* (Parker, 1910) (Halichondrida). The PCR-SSCP method allowed us to quickly detect different migration patterns of fragments which reflect DNA sequence diversity, both for individuals of different species and for individuals of the same population. We concluded that the PCR-SSCP method is: (1) useful to resolve differences at the species level; (2) simple, of low cost, fast, and non-radioactive; (3) excellent as a fast screening method for species identification, and might thus be a useful tool to discriminate sibling species and detect intragenomic variation without cloning, prior to sequence analysis.

KEY WORDS

Species identification, genetic diversity, internal transcribed spacers, nuclear ribosomal DNA, PCR-SSCP, Porifera.

INTRODUCTION

Studies of ribosomal DNA (rDNA) sequences have been used to infer phylogenetic history across a very broad spectrum of taxa, from Kingdoms and Phyla to closely related species and populations. The DNA sequence differences in the variable regions of the two internal transcribed spacers (ITS1 and ITS2) can be used to assess the structure and natural history of closely related taxa, to identify species or strains, to study hybridization, and as markers in population genetic studies (STOTHARD *et al.,* 1997; DAFFONCHIO *et al.,* 1998; EL TAI *et al.,* 2000; ZHU *et al.,* 2000). Amplification of the ITS via the polymerase chain reaction (PCR) is facilitated by conserved flanking regions in the 18S, 5.8S and 28S genes (HILLIS &

DIXON, 1991; HILLIS *et al.,* 1996). These sequences proved to be good molecular markers to separate species of sponges (WÖRHEIDE, 1998; LÔBO-HAJDU *et al.,* 2001; LOPEZ *et al.,* 2002; WÖRHEIDE *et al.,* 2002, 2003; DURAN *et al.,* 2004).

The single-strand conformation polymorphism (SSCP) method (ORITA *et al.,* 1989) has been used here to study levels of genetic variability in sponges. Material identified as *Cliona* aff. *celata* Grant, 1826 (Hadromerida) from Bahia, Rio de Janeiro and São Paulo States has been compared to another two species, *C. varians* (Duchassaing & Michelotti, 1864) (Rio Grande do Norte State) and *C. delitrix* Pang, 1973 (Bahia State), as a first step towards understanding genetic diversity within *Cliona*.

Additionally, preliminary observations (MORAES *et al.,* 1998) suggested a likely ecophenotypic explanation for the observed morphological plasticity in Brazilian *Hymeniacidon heliophila* (Parker, 1910) (Halichondrida) - size of papillae being negatively correlated with hydrodynamism, but apparently insensitive to degree of pollution or sedimentation. This species is reported for the Atlantic Ocean from the North Carolina coast (USA) to the Caribbean Sea, where it is widely distributed (DIAZ *et al.,* 1993) and also for the Southeastern Brazilian coast (Rio de Janeiro and São Paulo States) where, although being a common sponge, little is known about its morphological, cytological or molecular characteristics. No taxonomic description has been published for Brazilian specimens. In the North Atlantic Ocean, *H. heliophila* is considered a species with little morphological variations (DIAZ *et al.,* 1993). We decided to further test the SSCP method in searching for correlations between observed morphological plasticity and likely genetic diversity in *H. heliophila.*

The PCR-linked SSCP method has already been used successfully for species identification and in studies of population genetics in, for example, nematodes, Diptera, snails and fishes (GASSER *et al.,* 1997; KOEKEMOER *et al.,* 1999; SHARPE *et al.,* 1999; CLAPP *et al.,* 2000; SMALL & GOSLING, 2000; ASENSIO *et al.,* 2001). These studies have shown that PCR-linked SSCP is an accurate and sensitive method to detect genetic variation, and to assist in taxonomic investigation at levels ranging from populations to species.

We intend to determine genetic differences among five populations of *Hymeniacidon heliophila* and between three species of the genus *Cliona*, scattered along the Brazilian Coast, aiming at: 1) standardizing the PCR-linked SSCP method for identification of sponge species; and 2) determining at which taxonomic level the method is most useful to analyse genetic variability.

MATERIAL AND METHODS

Sponges

Samples were collected along the Brazilian coast (Fig. 1)through SCUBA and snorkelling, fixed in ethanol or frozen until DNA extraction (Tab. I). To include conspecific samples from different populations in the analysis, specimens of *C.* aff. *celata* were collected at Praia do Cabelo Gordo, São Sebastião, SP; Praia de Guarajuba, BA and Praia de Jõao Fernandes, Búzios, RJ. Specimens of *C. varians* were collected at Atol das Rocas, RN; Baía de Todos os Santos, Salvador, BA and Praia de Guarajuba, BA. All specimens of *Hymeniacidon* aff. *heliophila* were collected at Guanabara Bay, RJ (Praia Vermelha and Praia da Urca at Rio de Janeiro City and Praia de Boa Viagem and Praia de Itaipu at Niterói City) or São Sebastião, SP (Fig. 1).

Voucher specimens were deposited in the Porifera Collections of the Museu Nacional of the Universidade Federal do Rio de Janeiro (MNRJ).

Tab. I. Deposit number at UERJ, sponge species, collection sites, and voucher numbers. BA = Bahia State, PE = Pernambuco State, RJ = Rio de Janeiro State, RN = Rio Grande do Norte State, SP = São Paulo State, Brasil. MNRJ and UFRJPOR = Porifera Collections of Museu Nacional, Universidade Federal do Rio de Janeiro.

UERJ NUMBERS	SPECIES	COLLECTION SITES	VOUCHER NUMBERS
119	*Amphimedon viridis* Duchassaing & Michelotti, 1864	Praia do Sono, RJ	UFRJPOR 4747
106	*Aplysina fulva* (Pallas, 1766)	Abrolhos, BA	UFRJPOR 4699
006-008	*Cliona* aff. *celata* Grant 1826	Praia do Cabelo Gordo, São Sebastião, SP	not available
045-046	*Cliona* aff. *celata* Grant 1826	Praia de Guarajuba, BA	not available
052-055	*Cliona* aff. *celata* Grant 1826	Praia de Guarajuba, BA	not available
179	*Cliona* aff. *celata* Grant 1826	Praia de João Fernandinho, Búzios, RJ	not available
047-051	*Cliona varians* (Duchassaing & Michelotti, 1864)	Praia de Guarajuba, BA	not available
155-160	*Cliona varians* (Duchassaing & Michelotti, 1864)	Atol das Rocas, RN	not available
178	*Cliona dyorissa* Rützler, 1974	Praia de João Fernandinho, Búzios, RJ	not available
188-190	*Cliona delitrix* Pang, 1973	Baía de Todos os Santos, Salvador, BA	not available
319-330	*Hymeniacidon* aff. *heliophila* (Parker, 1910)	Praia Vermelha, Rio de Janeiro, RJ	*12 specimens
331-337	*Hymeniacidon* aff. *heliophila* (Parker, 1910)	Praia da Urca, Rio de Janeiro, RJ	*7 specimens
338-344	*Hymeniacidon* aff. *heliophila* (Parker, 1910)	Praia de Boa Viagem, Niterói, RJ	*7 specimens
345-353	*Hymeniacidon* aff. *heliophila* (Parker, 1910)	Praia de Itaipu, Niterói, RJ	*9 specimens
354-362	*Hymeniacidon* aff. *heliophila* (Parker, 1910)	Praia do Cabelo Gordo, São Sebastião, SP	*9 specimens
002	*Mycale* aff. *americana* Van Soest, 1984	São Sebastião, SP	MNRJ 1584
009	*Mycale escarlatei* Hajdu, Zea, Kielman & Peixinho, 1995	São Sebastião, SP	UFRJPOR 4451
013	*Mycale laxissima* Duchassaing & Michelotti, 1864	São Sebastião, SP	MNRJ 1027
099	*Mycale microsigmatosa* Arndt, 1927	São Sebastião, SP	MNRJ 1331
015	*Mycale magnirhaphidifera* van Soest, 1984	São Sebastião, SP	MNRJ 1330
136	*Mycale arcuiris* Lerner & Hajdu, 2002	São Sebastião, SP	MNRJ1942
100	*Mycale angulosa* (Duchassaing & Michelotti, 1864)	Parati, RJ	UFRJPOR 4743
094	*Dragmacidon reticulatus* (Riedley & Dendy, 1886)	São Sebastião, SP	MNRJ 289
105	*Topsentia ophiraphidites* (De Laubenfels, 1934)	Fernando de Noronha, PE	UFRJPOR 4779

*Several individuals were collected at the same site.

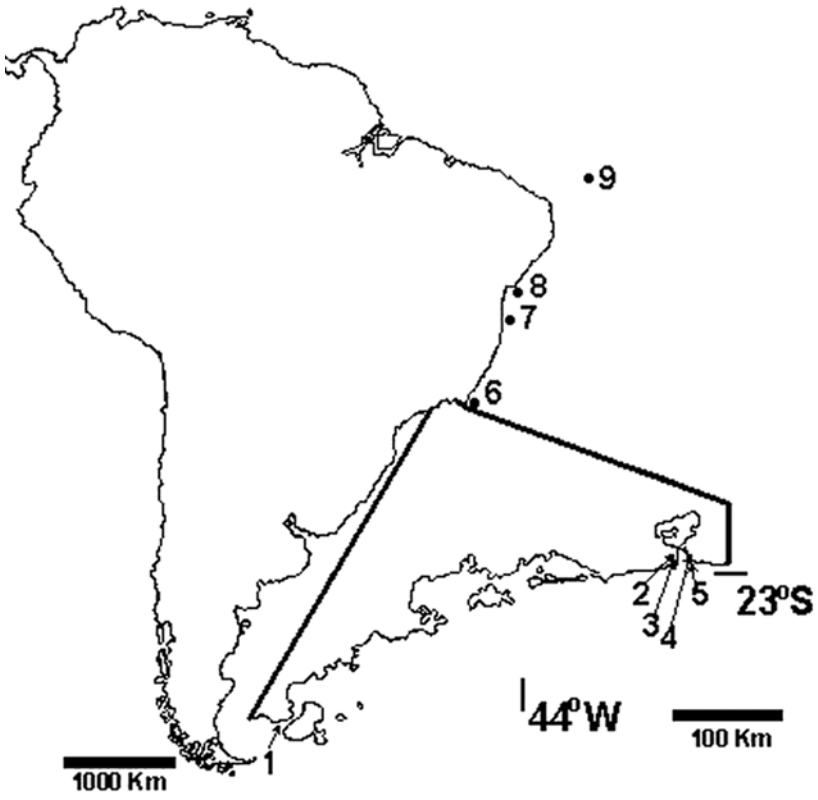

Fig. 1. Collection sites for *Cliona* species and *Hymeniacidon heliophila* specimens: 1 = Praia do Cabelo Gordo, São Sebastião, SP; 2 = Praia Vermelha, Guanabara Bay, RJ; 3 = Praia da Urca, Guanabara Bay, RJ; 4 = Praia de Boa Viagem, Guanabara Bay, RJ; 5 = Praia de Itaipú, Guanabara Bay, RJ; 6 = Praia de Jõao Fernandes, Búzios, RJ; 7 = Baía de Todos os Santos, Salvador, BA; 8 = Praia de Guarajuba, BA; 9 = Atol das Rocas, RN.

DNA extraction

After careful dissection to remove macroscopic symbionts on an ice-cooled Petri dish, specimens were ground with a rod in a mortar with 1:5 (weight:volume) solution of 4M guanidine hidrochloride, 50mM Tris-HCl pH 8.0, 0.05 M EDTA, 0.5 % sodium-N'-lauroylsarcosine and 1 % ß-mercaptoethanol. The suspension was incubated at 50° C for 1 h and centrifuged at 3000 g / 20 min. The supernatant was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and nucleic acids were precipitated with 2 volumes of ethanol. The pellet was washed in 70 % ethanol and air dried. The dried pellet was dissolved in sterile water plus 20 µg/ml RNAse A (GIBCO BRL) and incubated at 37° C for 2 h. The DNA concentration was estimated in 0.8 % agarose gels in TBE 0.5X (50 mM Tris Base, 50 mM boric acid, 1 mM EDTA) by comparison with solutions of known

concentrations of lambda bacteriophage genomic DNA (GIBCO BRL) (10 ng/µl, 20 ng/µl, 50 ng/µl, 100 ng/µl), incubated with 0.6 µg/ml ethidium bromide and visualized under UV light.

PCR

The ITS regions were amplified using the pair of primers 18SFow: 5´-TCATTTAGAGG AAGTAAAAGTCG-3´ plus 5.8SRev: 5´-GCGTTCAAAGACTCGATGATTC-3´ and 5.8SFow: 5´-GAATCATCGAGTCTTTGAAGCC-3´ plus 28SRev: 5´-GTTAGTTTCTTTT CCTCCGCTT-3´, for ITS1 and ITS2 respectively. Primers used in this study were designed according to a multiple sequence alignment using Genebank sequences aligned with Clustal W (THOMPSON *et al.,* 1994). We used the nucleotides present in the sequence of *Hymeniacidon heliophila* (ODORICO & MILLER, 1997a) at positions where more than one type of nucleotide was found. Each PCR amplification reaction mixture of 50 µl contained 10 ng of genomic DNA, reaction buffer (10 mM KCl, 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 0.1 % Triton-X-100, 100 mg/ml gelatin), 3 mM $MgSO_4$, 200 µM dNTPs (Amersham Pharmacia), 80 ng of each primer and 1 unit of *Pfu* DNA polymerase (produced by R.M. Albano). Amplification was carried out in a DNA thermal cycler (M.J. Research PTC-100). An initial denaturation step of 5min at 96° C was followed by 35 cycles of 30 s at 94° C, 45 s at 52° C and 1 min at 72° C, with an additional final step of 5 min at 72° C. The amplified bands were separated by electrophoresis on 2 % agarose gels in TBE 0.5X. The size of amplified fragments was estimated by comparison with standard DNA markers.

SSCP

The samples were subjected to electrophoresis in an 8 % acrylamide (50 % or 99:1 acrylamide:bis-acrylamide) non-denaturing gel for SSCP (ORITA *et al.,* 1989). Each 3µl of PCR product is added of 5 µl denaturing buffer (NaOH 10 mM, formamide 95 %, bromofenol blue 0.05 % and xyleno cyanol 0.05 %), the sample mixture is boiled during 5 min at 95° C and immediately after this step, placed on ice to maintain the denatured single strand state. This mixture is applied on gel and run for 18 h in a cold chamber under an electric field of 15 V/cm. The gel is visualized after silver nitrate impregnation (BASSAM *et al.,* 1991), dried and scanned.

SSCP data interpretation and analysis

Relationships between SSCP profiles were explored by comparing the presence (1) or absence (0) of shared variants using PHYLIP software (FELSENSTEIN, 1993), which allowed the calculation of the genetic distances and the construction of a phenogram of similarity between taxa through the MEGA software (KUMAR *et al.,* 1993).

RESULTS AND CONCLUSIONS

The sizes of the DNA fragments yielded by PCR amplification of the ITS1, ITS2 and ITS1-5.8S-ITS2 in *Hymeniacidon* aff. *heliophila* are shown in Fig. 2. For this species, the ITS1 fragment had approximately 350 base pairs (bp), the ITS2 had 300 bp and both ITS plus the 5.8S rRNA gene had close to 800 bp. Fig. 3 shows the ITS1 fragment amplified from *H. heliophila* individuals out of two populations. On agarose gel, there was no clear difference in size when DNA fragments from the same species were compared, neither between nor within populations. However, both ITS1 and ITS2 fragments vary in sizes between different sponge species. An example can be seen in Fig. 4.

The SSCP technique allows the distinction of specimens with the limit of a single nucleotide change. The method is based on the denaturation of PCR products

(double stranded) yielding single stranded DNA, which folds through internal base pairing assuming tertiary structure (conformations). These structures migrate on polyacrylamide gels on a way that correlates with the number of mutations (substitutions, indels) accumulated in an individual, population or species.

Fig. 2. PCR products on a 2 % agarose gel. Lane 1 = molecular weight DNA standard of lambda phage digested with *EcoRI* and *HindIII*, from top to bottom: 2027, 1904, 1584, 1375, 947, 831 and 564 base pairs (bp). Lane 2 = ITS1; lane 3 = ITS2 and lane 4 = ITS1+5.8S+ITS2 amplified from *H. heliophila* BV3. Where: BV = Praia da Boa Viagem, Niterói, RJ. Lane 5 = molecular weight DNA standard of pBlueScript SK plasmid digested with *HinfI*: 1.077, 517, 456, 396 and 356 bp.

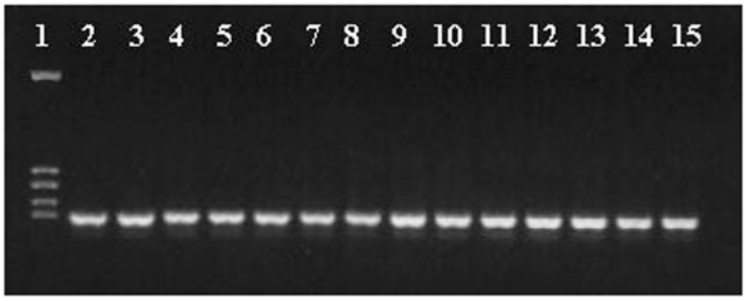

Fig. 3. Inter- and intraspecific variation of *H. heliophila* ITS1 fragments amplified by PCR on a 2 % agarose gel. Lane 1 = molecular weight DNA standard of pBlueScript SK plasmid digested with *HinfI*. Lane 2 = PV1; lane 3 = PV2; lane 4 = PV3; lane 5 = PV5; lane 6 = PV6; lane 7 = PV7; lane 8 = PV8; lane 9 = U1; lane 10 = U2; lane 11 = U3; lane 12 = U4; lane 13 = U5; lane 14 = U6; lane 15 = U7. Where: PV = Praia Vermelha, RJ and U = Praia da Urca, RJ.

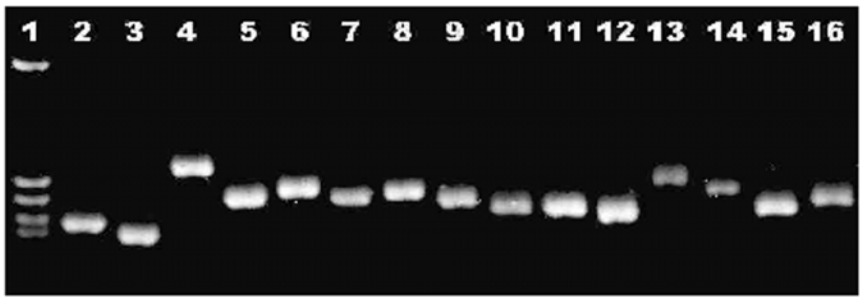

Fig. 4. Interspecific variation of ITS1 PCR products of different demosponge genera in a 2 % agarose. Lane 1 = molecular weight DNA standard of pBlueScript SK plasmid digested with *HinfI*. Lane 2 = *Hymeniacidon heliophila*; lane 3 = *Mycale* aff. *americana*.; lane 4 = *Mycale arcuiris*; lane 5 = *Mycale escarlatei*; lane 6 = *Mycale magnirhaphidifera*; lane 7 = *Mycale angulosa*; lane 8 = *Mycale laxissima*; lane 9 = *Mycale microsigmatosa*; lane 10 = *Cliona* aff. *celata*; lane 11 = *C. varians*; lane 12 = *C. delitrix*; lane 13 = *Dragmacidon reticulatus*; lane 14 = *Aplysina fulva*; lane 15 = *Amphimedon viridis*; lane 16 = *Topsentia ophirhaphidites*.

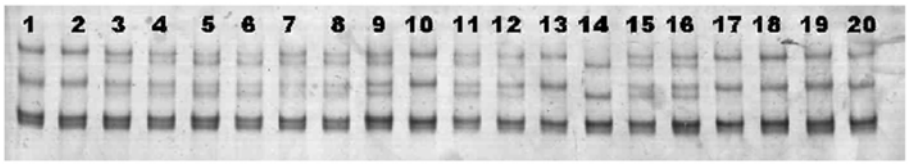

Fig. 5. Intraspecific variation of ITS1 in *H. heliophila* shown on an 8 % SSCP-PAGE gel. Lanes 1 - 4 = Praia Vermelha, RJ; lanes 5 - 8 = Praia da Urca, RJ; lanes 9 - 12 = Praia da Boa Viagem, RJ; lanes 13 - 16 = Praia de Itaipu, RJ; lanes 17 - 20 = Praia do Cabelo Gordo, São Sebastião, SP.

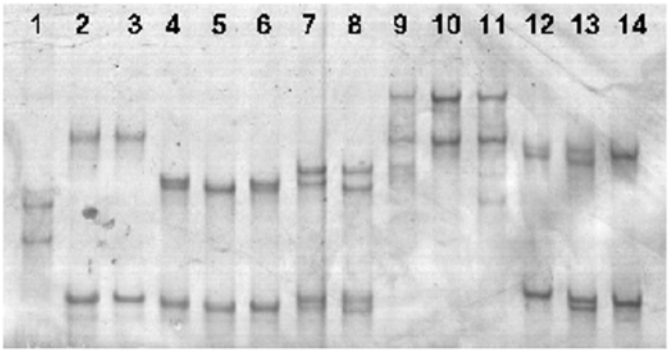

Fig. 6. Intra- and interspecific variation of ITS2 within the genus *Cliona* on an 8 % SSCP-PAGE gel. Lane 1 = *Cliona dyorissa*; lanes 2 - 3 = *Cliona delitrix*; lanes 4 - 8 = *Cliona varians* and lanes 9 - 14 = *Cliona* aff. *celata*. Conspecific populations of *C. varians*: Praia de Guarajuba, BA (lane 4), Baía de Todos os Santos, BA (lane 5), Atol das Rocas, RN (lanes 6 - 8) and of *C.* aff. *celata*: Praia de Jõao Fernandes, Búzios, RJ (lane 9), Praia do Cabelo Gordo, São Sebastião, SP (lanes 10 - 11), Praia de Guarajuba, BA (lanes 12 - 14).

The gel in Fig. 5 presents the migration pattern of ITS1 fragments in different populations and between individuals of the same population of *H.* aff. *heliophila*. It was noted that the migration pattern presented intra- and interpopulation variation. The specimens on lanes 1, 2, 10, 13, 17, 18, 19 and 20 had one pattern of SSCP variant, while specimens on lanes 3, 4, 5, 6, 7, 8, 9, 11, 12, 15 and 16 had an alternative pattern. The specimen on lane 14 had a third pattern or SSCP variant. Each SSCP variant corresponds to an allele.

These alleles were used to generate a similarity phenogram. Only one tree was obtained for the 20 individuals and three characters (SSCP variants), showing unresolved polytomies, which indicate that the five populations of *H.* aff. *heliophila* cannot be separated in distinct groups (data not shown).

It is expected that due to the process of concerted evolution the ITS should be homogeneous in sequence within an individual. Molecular processes such as unequal crossing-over, gene conversion and transposition have been proposed as mechanisms that not only generate variation in multigene arrays on which natural selection can operate, but also as mechanisms by which mutations spread through a multigene family (homogenisation) and become fixed in a population/species (molecular drive) (FRITZ *et al.,* 1994 and references therein). However, the degree of differentiation observed within a species is a balance between those processes that generate variability and those that lead to homogenisation and fixation. In all cases reported so far where intraindividual variation has been looked for within ITS1 and ITS2, it has been found to some degree. This includes studies on beetles (VOGLER & DESALLE, 1994), crayfish (HARRIS & CRANDALL, 2000), coral *Acropora* (ODORICO & MILLER, 1997b), and more recently in the sponge *Crambe crambe* (DURAN *et al.,* 2004). As shown in Fig. 5, it is possible to distinguish three pairs of double bands in some lanes, for example 5 to 8, that most likely represent different types of intragenomic variants rather than distinct alleles. In repetitive markers such as ITS, it is usually impossible to distinguish between homozygous or heterozygous individuals and more than one different copy may occur in diploid individuals. The next step will involve the use of a more direct method, where individual variants should be cloned and sequenced.

The ITS1 fragment is less variable. It presented less SSCP variants than the ITS2 fragment of *H.* aff. *heliophila* in the five populations analysed (data not shown).

There is a clear difference between the SSCP patterns of each *Cliona* species, but the pattern is generally similar between individuals of the same species (Fig. 6). In the ITS2 analysis of *Cliona* species the SSCP patterns were also generally similar among individuals of the same species in *C. varians* and *C. delitrix*, and among individuals of the same populations in *C.* aff. *celata* (Fig. 6). *Cliona* aff. *celata* showed intraspecific variation both among populations (lanes 9 - 14 in Fig. 6) and within a single population (lanes 12 - 14, Fig. 6). The possibility that different populations of *C.* aff. *celata* represent distinct biological species is being investigated through DNA sequencing of the ITS1 and ITS2 regions.

Our results show that it is possible to distinguish different patterns of SSCP for different species and populations of sponges. The PCR-SSCP revealed different migration patterns of fragments for individuals of different species and for individuals of the same population, and might thus be a useful tool to discriminate sibling sponge species and detect intragenomic variation without cloning. This

technique is excellent as a fast screening method for sponge species identification prior to sequence analysis. To assess the number and type of mutations, experiments of dideoxynucleotide sequencing of the ITS1 + 5.8S + ITS2 fragment is mandatory. These DNA sequence differences can be used to infer phylogeny among closely related taxa and as markers in population genetic studies.

We have been able to separate species of the genus *Cliona* on the basis of SSCP variants, which may be a useful additional character when morphology is inconclusive. The intrapopulation diversity of the SSCP variants in the five populations of *Hymeniacidon* aff. *heliophila* was as high as the interpopulation variability. This suggests that all five populations belong to the same species. There is gene flow between them, in spite of the different morphotypes, which are most probably correlated to ecological and/or environmental influence.

The ITS sequences proved to be good molecular markers to separate species of sponges of further genera, viz. *Mycale* Gray, 1867 (Poecilosclerida), *Aplysina* Nardo, 1834 (Verongida) and *Geodia* Lamarck, 1815 (Astrophorida), as reported previously by our group (LÔBO-HAJDU *et al.,* 2001).

Major advantages in using SSCP techniques include their simplicity and low cost (SUNNUCKS *et al.,* 2000). Moreover, the technique does not make use of radioactive probes and does not require large amounts of DNA.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr. E. Hajdu and Dr. G. Muricy (Museu Nacional-UFRJ, Rio de Janeiro, RJ, Brasil) for collecting and identifying the specimens. E. Hajdu is further thanked for improving clarity of explanation in the final draft of this manuscript. This work was carried out with financial assistance from Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

REFERENCES

ASENSIO L., GONZÁLEZ I., FERNÁNDEZ A., RODRIGUEZ M.A., HERNÁNDEZ P.E., GARCÍA T., MARTÍN R., 2001 - PCR-SSCP: A simple method for the authentication of grouper (*Epinephelus guaza*), wreck fish (*Polyprion americanus*), and nile perch (*Lates niloticus*) fillets. *J. Agric. Chem.,* **49**: 1720-1723.

BASSAM B.J., CAETANO-ANOLLES G., GRESSHOFF P.M., 1991 - Fast and sensitive silver staining of DNA in polyacrilamide gels. *Anal. Biochem.,* **196**: 80-83.

CLAPP J.P., STOEL C.D. VAN DER, PUTTEN W.H. VAN DER, 2000 - Rapid identification of cyst (*Heterodera* spp., *Globodera* spp.) and root-knot (*Meloidogyne* spp.) nematodes on the basis of ITS2 sequence variation detected by PCR-single-strand conformation polymorphism (PCR-SSCP) in cultures and field samples. *Mol. Ecol.,* **9**: 1223-1232.

DAFFONCHIO D., BORIN S., CONSOLANDI A., MORA D., MANACHINI P.L., SORLINI C., 1998 - 16S-23S rRNA internal transcribed spacers as molecular markers for the species of the 16S rRNA group I of the genus *Bacillus*. *FEMS Microbiol. Lett.,* **163**: 229-236.

DIAZ M.C., POMPONI S.A., SOEST R.W.M. VAN, 1993 - A systematic revision of the Central West Atlantic Halichondrida (Demospongiae, Porifera). Part III: Description of valid species. *Sci. Mar.,* **57**: 283-306.

DURAN S., GIRIBET G., TURON X., 2004 - Phylogeographical history of the sponge *Crambe crambe* (Porifera, Poecilosclerida): range expansion and recent invasion of the Macaronesian islands from the Mediterranean Sea. *Mol. Ecol.,* **13**: 109-122.

EL TAI N.O., OSMAN O.F., EL FARI M., PRESBER W., SCHÖNIAN G., 2000 - Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of *Leishmania donovani* spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. *Trans. R. Soc. Trop. Med. Hyg.,* **94**: 575-579.

FELSENSTEIN J., 1993 - PHYLIP, Phylogenetic Inference Package. Computer Program and Manual. University of Washington, Washington.

FRITZ G.N., CONN J., COCKBURN A., SEAWRIGHT J., 1994 - Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovari* (Diptera: Culicidae). *Mol. Biol. Evol.,* **11**: 406-416.

GASSER R.B., MONTI J.R., ZHU X., CHILTON N.B., HUNG G.-C., GULDBERG P., 1997 - Polymerase chain reaction-linked single-strand conformation polymorphism of ribosomal DNA to fingerprint parasites. *Electrophoresis,* **18**: 1564-1566.

HARRIS D.J., CRANDALL K.A., 2000 - Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): implications for phylogenetic and microsatellite studies. *Mol. Biol. Evol.,* **17**: 284-291.

HILLIS D.M., DIXON M.T., 1991 - Ribosomal DNA: Molecular evolution and phylogenetic inference. *Q. Rev. Biol.,* **66**: 411-453.

HILLIS D.M., MORITZ C., MABLE B.K., 1996 - Molecular Systematics, 2nd. Edition. Sinauer Associates, Sunderland, Mass., 655 pp.

KOEKEMOER L.L., LOCHOUARN L., HUNT R.H., COETZEE M., 1999 - Single-strand conformation polymorphism analysis for identification of four members of the *Anopheles funestus* (Diptera: Culicidae) group. *J. Med. Entomol.,* **36**: 125-130.

KUMAR S., TAMURA K., NEI M., 1993 - MEGA: Molecular Evolutionary Genetics Analysis, Version 1.02. Pennsylvania State University, University Park, PA.

LÔBO-HAJDU G., SALGADO A., GUIMARÃES A.C.R., LAMARÃO F.R.M., ALBANO R.M., SILVA C.M.M., HAJDU E., MURICY G., 2001 - Use of PCR-linked single strand conformation polymorphism of rDNA ITS sequences to distinguish species of Porifera. In: IX COLACMAR, Noveno Congreso Latinoamericano sobre Ciencias del Mar, San Andrés Isla, Colômbia: 158 and short communication on CD.

LOPEZ J.V., PETERSON C.L., WILLOUGHBY R., WRIGHT A.E., ENRIGHT E., ZOLADZ S., REED J.K., POMPONI S.A., 2002 - Characterization of genetic markers for *in vitro* cell line identification of the marine sponge *Axinella corrugata*. *J. Hered.,* **93**: 27-36.

MORAES F.C., HAJDU G.L., SALGADO A., ALBANO R.M., MURICY G., 1998 - Variações Ecológicas, Morfológicas e Moleculares na Esponja *Hymeniacidon heliophila* (Parker, 1910) (Porifera, Halichondriida). *An. XXII Congr. Bras. Zool.,* Recife, Brasil: 3 (abstract 009).

ODORICO D.M., MILLER D.J., 1997a - Internal and external relationships of the Cnidaria: implications of primary and predicted secondary struture of the 5' - end of the 23S - like rDNA. *Proc. R. Soc. London B,* **264**: 77-82.

ODORICO D.M., MILLER D.J., 1997b – Variation in the ribosomal internal transcribed spacers and 5.8S rDNA among five species of *Acropora* (Cnidaria, Scleractinia): patterns of variation consistent with reticulate evolution. *Mol. Biol. Evol.,* **14**: 465-473.

ORITA M., SUZUKI Y., SEKIYA T., HAYASHI K., 1989 - Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics,* **5**: 874-879.

SHARPE R.G., HIMS M.M., HARBACH R.E., BUTLIN R.K., 1999 - PCR-based methods for identification of species of the *Anopheles minimus* group: allele-specific amplification and single-strand conformation polymorphism. *Med. Vet. Entomol.,* **13**: 265-73.

SMALL M.P., GOSLING E.M., 2000 - Species relationships and population structure of *Littorina saxatilis* Olivi and *L. tenebrosa* Montagu in Ireland using single-strand conformation polymorphisms (SSCPs) of cytochrome-b fragments. *Mol. Ecol.,* **9**: 39-52.

SUNNUCKS P., WILSON A.C.C., BEHEREGARY L.B., ZENGER K., FRENCH J., TAYLOR A.C., 2000 - SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. *Mol. Ecol.,* **9**: 1699-1710.

STOTHARD J.R., FRAME I.A., MILES M.A., 1997 - Use of polymerase chain reaction-based single strand conformational polymorphism and denaturing gradient gel electrophoresis methods for detection of sequence variation of ribosomal DNA of *Trypanossoma cruzi*. *Int. J. Parasit.,* **27**: 339-343.

THOMPSON J.D., HIGGINS D.G., GIBSON T.J., 1994 - Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.,* **22**: 4673-4680.

VOGLER A., DESALLE R., 1994 - Evolution and phylogenetic information content of the ITS-1 region in the tiger beetle *Cicindela dorsalis*. *Mol. Biol. Evol.,* **11**: 393-405.

WÖRHEIDE G., 1998 - The reef cave dwelling ultraconservative coralline Demosponge *Astrosclera willeyana* Lister 1900 from the Indo-Pacific. Micromorphology, ultrastructure, biocalcification, isotope record, taxonomy, biogeography, phylogeny. *Facies,* **38**: 1-88.

WÖRHEIDE G., DEGNAN B.M., HOOPER J.N.A., REITNER J., 2003 - Phylogeography and taxonomy of the Indo-Pacific reef cave dwelling coralline demosponge *Astrosclera willeyana* - new data from nuclear internal transcribed spacer sequences. In K.M. Moosa, S. Soemodihardjo, A. Nontji, A. Soegiarto, K. Romimohtarto, Sukarno and Suharsona (eds), *Proc. 9th Internat. Coral Reef Symp.,* Jakarta: 339-346.

WÖRHEIDE G., HOOPER J.N.A., DEGNAN B.M., 2002 - Phylogeography of western Pacific *Leucetta ´*chagosensis*´* (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.

ZHU X., D'AMELIO S., PAGGI L., GASSER R.B., 2000 - Assessing sequence variation in the internal transcribed spacers of ribosomal DNA within and among members of the *Contracaecum osculatum* complex (Nematoda: Ascaridoidea: Anisakidae). *Parasitol. Res.,* **86**: 677-683.