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Analysis of intragenomic variation of the rDNA internal transcribed spacers (ITS) in Halichondrida (Porifera: Demospongiae)

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Three species of sponges, *Axinella aruensis*, *Phakellia* sp. and *Acanthella cavernosa*, were selected to investigate the extent of intragenomic variation (IGV) in the internal transcribed spacers (ITS-1 and ITS-2) of the ribosomal DNA within the order Halichondrida. The IGV was detected in both the spacers and in the three species. At least three non-identical sequences were found in any single individual with the number of polymorphic sites within species ranging from 6 to 77. Uncorrected distance values up to 29% were observed in the ITS-2 of *Axinella aruensis*. The levels of polymorphism found in this species are the highest reported for Porifera and comparable to those observed in hybrid species of scleractinean corals. Analyses of ITS sequences from multiple clones from individuals of Halichondrida are strongly recommended if these genetic markers are to be used to depict phylogenetic and/or phylogeographic relationships of closely related species and their populations. Data from other nuclear and mitochondrial markers should be used to complement conclusions derived from studies based on ITS sequences.

INTRODUCTION

Sponge classification currently relies on the use of morphological characters, mainly skeletal, although they are complemented in some groups with characteristics of the aquiferous system (see Hooper & van Soest, 2002). These characters, however, in many instances are either autapomorphic or non-homologous and become inappropriate or insufficient to establish well supported phylogenetic relationships of sponges at most levels of classification (see review by Boury-Esnault, 2006).

To support sponge classification with robust phylogenetic hypotheses, researchers have included biochemical (van Soest & Braekman, 1999; Castellanos et al., 2003) and genetic (see below) characters in their analyses.

Several molecular markers, both nuclear and mitochondrial, have been used in sponge phylogenetics. Complete and partial sequences of the small (18S) and large (28S) ribosomal DNA (rDNA) are currently used by most researchers to reconstruct phylogenies at different systematic levels (see review by Boury-Esnault, 2006). Other nuclear genes, such as elongation factor-1 alpha (Erpenbeck et al., 2005b), the intron-2 of tubulin (Schroder et al., 2003), the heat shock protein 70 (Hsp70 cDNA) family (Borchiellini et al., 1998), the second intron of the ATP synthetase beta subunit (Bentlage & Wörheide, 2007) or homeobox sequences (Richelle-Maurer et al., 2006 and references therein) have recently been used to study basal relationships within Porifera.

The mitochondrial cytochrome oxidase I (COI), generally used in other metazoans for lower level phylogenies and biogeographical studies, has been included in sponge studies to resolve relationships within species complexes (Duran et al., 2004b; Erpenbeck et al., 2005c; Nichols, 2005; Wörheide, 2006). These studies show that in most cases this gene is too conservative to resolve relationships at the level of species and below (but see Duran & Rützler, 2006).

Phylogeny at lower taxonomic levels and phylogeography of many eukaryotes (e.g. Van Oppen et al., 2000; Lopez et al., 2002; Coleman, 2003 and references therein; Vollmer & Palumbi, 2004) especially plants (see review by Alvarez & Wendel, 2003) are often based on sequences from the rDNA ITS-1 and ITS-2. In sponges, however, the use of this marker for studies at this systematic level is limited to a few studies (e.g. Lopez et al., 2002; Wörheide et al., 2002a,b; Duran et al., 2004b; Lobo-Hajdu et al., 2004; Nichols & Barnes, 2005; Schmitt et al., 2005). Most of these studies have shown that the ITS spacers have an appropriate rate of evolution to study relationships of sponges at the level of species and for the differentiation of species complexes, nevertheless the use of this region in phylogenetic analyses needs to be carefully considered.

The ribosomal genes encoding the small (5.8S, 18S) and large (28S) subunits and its spacers (ITS-1 and ITS-2) are present within the genome in multiple copies that do not evolve independently. Generally, mutations occurring in one of these repeats are homogenized, or 'copied' to the rest of the gene family members, through mechanisms of concerted evolution such as unequal crossing over and high-frequency gene conversion, but this may not always be the case (Page & Holmes, 1998). As a result, divergent paralogues or non-homologous sequences can be found within one individual

Table 1. *Sponge samples used in this study, corresponding NTM registration numbers, locality and collection data. All samples were collected from localities in Northern Territory, Australia.*

NTM No.	Species	Locality and collection data
Z004486	*Phakellia* sp.	Raft Point, Bynoe Harbour, 12°37.682' S 130°32.175'E, 5–8 m depth, 26 June 2003
Z004487	*Acanthella cavernosa*	Dawson Rock, 3 km SSE of Rankin Point, Bynoe Harbour, 12°42.207'S 130°35.459'E, 5 m depth, 1 June 2005
Z004488	*Phakellia* sp.	Same as above
Z004489	*Acanthella cavernosa*	Same as above
Z004490	*Axinella aruensis*	Stephen's Rock, West Arm, Darwin Harbour, NT, Australia 12°29.1667'S 130°47.19'E, NT, 5 m depth, 8 May 2006
Z004491	*Axinella aruensis*	Same as above

genome and potentially confound phylogenetic inferences especially at intraspecific levels (see review by Alvarez & Wendel, 2003; Wörheide et al., 2004).

The IGV in the ITS spacers of rDNA of sponges has been investigated using two molecular methods. Single stranded conformation polymorphism (SSCP), has been used to indirectly assess IGV in individuals of *Axinella corrugata* (Lopez et al., 2002), *Hymeniacidon* aff. *heliophila*, *Cliona* aff. *celata* and *Cliona varians* (Lobo-Hajdu et al., 2004). Cloning and sequencing of PCR products containing the ITS region, which is a more direct method to assess IGV, has been used for: *Aplysina aerophoba* (one individual); in *Crambe crambe* (nine populations, 8–12 individuals/population), *Placospongia* sp. (28 individuals, 12 populations), *Spirastrella harmani* (two individuals), *S. sabogae* (one individual) and *Polymastia* sp. (one individual) (Duran et al., 2004a; Nichols & Barnes, 2005; Schmitt et al., 2005). Wörheide et al. (2004) however, studied in more detail the extent of IGV levels in a variety of demosponge taxa: Verticillitida, *Vaceletia* sp., Dictyoceratida, *Dysidea* sp.; Hadromerida, 7 spp., including the *Prosuberites laughlini* species complex; Agelasida, 2 spp.; Calcarea, 8 spp., including the *Leucetta chagosensis* species complex; and Hexactinellida, 2 spp. In most of these studies IGV did not seem to affect the phylogenetic relationships of the studied taxa at the species level but in some cases, paralogous sequences that could mislead conclusions regarding the structure of the studied populations were detected (i.e. *Placospongia* and *Prosuberites laughlini* Nichols, 2005; Wörheide et al., 2004, respectively). Wörheide et al. (2004) concluded that levels of IGV in sponges are heterogeneous and cannot be predicted by taxonomy, and that significant amounts of IGV will have implications for relationships at the population level.

Ongoing studies on northern Australian sponges of the order Halichondrida (B. Alvarez, unpublished data) will consider the use of ITS DNA sequences, among other markers, to distinguish species and test the monophyly of allegedly polyphyletic genera *Axinella*, *Phakellia* (Axinellidae) and *Acanthella* (Dictyonellidae) (Alvarez et al., 2000). According to Lopez et al. (2002) the ITS rDNA has enough signal to study phylogenetic relationships and biogeographical patterns of axinellid-like sponges and therefore seems an appropriate choice for these studies.

The levels or IGV of the ITS ribosomal spacers in sponges of the order Halichondrida, however, with the exception of the data generated by SSCP on *Axinella corrugata* (Lopez et al., 2002) and *Hymeniacidon* aff. *heliophila* (Lobo-Hajdu et al., 2004), are so far unknown. Following the recommendations of Wörheide et al. (2004) we investigated selected species of Halichondrida to determine whether or not intra-individual variation of the rDNA spacers is present and its significance in affecting the conclusions of future phylogenetic analyses based on these sequences.

MATERIALS AND METHODS

Collection and preparation of samples

Samples of two individuals of *Axinella aruensis* (Hentschel, 1912), an undescribed species of *Phakellia* and *Acanthella cavernosa* (Dendy, 1922) were collected in rocky reef habitats at Bynoe Harbour, and Darwin Harbour, Australia, by SCUBA diving. Small pieces were cut from each sponge, washed with seawater and preserved in 100% ethanol. The remaining sponge tissue was preserved in 70% ethanol, and deposited at the Museum and Art Gallery of the Northern Territory (NTM). Corresponding voucher numbers and, locality data are presented in Table 1.

DNA extraction and PCR

Small pieces of the preserved sponge tissue were rinsed with high-pure water and macerated using a mortar and pestle. Approximately 10 mg of macerated tissue was used for DNA extraction using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions with the lysis step extended overnight.

Ribosomal ITS-1 and 2 were amplified with the primer sequences 5'GTC CCT GCC CTT TGT ACA CA3' (RA2) and 5'CCT GGT TAG TTT CTT TTC CTC CGC3' (ITS 2.2) (Wörheide et al., 2004). The amplified region also contained the 3' region of the 18S gene and 5' of the 28S gene.

The PCR amplifications were performed in a 50 µl reaction containing 2–20 ng of genomic DNA, 1× reaction buffer (67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$; gelatine at 2 µg/µl; 0.45% Triton X-100, 1.5 mM $MgCl_2$), 100 µM each dATP, dCTP, dGTP and dTTP, 0.4 µM of each primer, and 1.25 units of Taq polymerase (Geneworks).

The PCR conditions were an initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 55°C for 90 s and 72°C for 2 min followed by a final extension step at 72°C for 5 min. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen).

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Table 2. *Position of different rDNA regions in the alignments of the studied individuals. Sequences identified by Accession numbers AJ705046 (for* Phakellia *sp. and* Axinella aruensis*) and AF340018 (for* Acanthella cavernosa*) were used to identify the different regions.*

	18S	ITS-1	5.8S	ITS-2	28S
Phakellia sp.					
Z004486	1–188	189–469	470–663	664–814	815–897
Z004488	1–188	189–491	492–660	661–824	825–906
Acanthella cavernosa					
Z004487	1–188	189–467	468–651	652–821	822–874
Z004489	1–188	189–473	474–653	654–829	829–876
Axinella aruensis					
Z004490	1–188	189–484	485–637	638–788	789–862
Z004491	1–188	189–496	497–643	644–800	801–879

Table 3. *Average base pair (bp) sequence length, maximum and average uncorrected* p*-distances observed among sequenced clones. Maximum values in bold.*

Individual	Average Length (bp)	Average *p*-distance	Maximum *p*-distance
Phakellia sp.			
Z004486	897	0.00596	0.00893
Z004488	848	0.00565	0.01228
Axinella aruensis			
Z004490	848	0.02625	0.03565
Z004491	848	**0.05331**	**0.07731**
Acanthella cavernosa			
Z004487	873	0.00787	0.01375
Z004489	874	0.00533	0.01028

Cloning

Purified PCR products of each individual (Table 1) were ligated into pGEM-T easy vector (Promega). The ligated products were precipitated, resuspended in sterile distilled water and transformed in XL 1-Blue MRF' Electro competent cells (Stratagene) using a gene pulser (Bio-Rad) set at 25 μF, 2.5kV and 200Ω. Bacteria were grown on agar plates with ampicillin X-gal (X-gal, dimethyl formamide at 20 mg/ml) and isopropyl-beta-D-thiogalactopyranoside (IPTG) to select colonies with recombinant plasmids (i.e. white colonies). To identify colonies with the correct-sized insert, 10 to 15 white colonies were screened by PCR and the resulting products were separated by electrophoresis through a 1% agarose gel. At least six colonies/individual, containing an insert of 850–900 bp were re-grown in Luria Bertani +ampicillin broth (Bacto-Tryptone, yeast extract and sodium chloride) overnight. Plasmid DNA was extracted from this culture using a QIA-quick mini-prep kit (Qiagen).

The PCR products of each individual sample were cloned separately to avoid cross contamination during the process.

Sequencing

Approximately 400–600 ng of cloned DNA were sequenced using M13 pGEM-T vector primers with the ABI Big Dye Terminator reaction Kit (BDT v. 3.1) on a genetic analyser (ABI GA3130XL) at the Bioscience North Australia sequencing facility. Given that the optimal number of clones to study intragenomic polymorphism of the selected species is not known and based on available resources at least six clones of each individual were sequenced in both directions.

Sequences of each cloned PCR product were assembled using the software MacVector v. 9.0 (Accelrys Software Inc.) and compared to other sequences from the National Centre for Bioinformatics (NCBI), http://www.ncbi.nlm.nih.gov/database using the Basic Local Alignment Search Tool (BLAST) to confirm that they were of sponge origin.

Sequences were deposited in the GenBank under Accession numbers EF646765–EF646800.

Data analyses

The assembled sequences of all six clones of each individual were aligned using ClustalW (Thompson et al., 1994) in MacVector v. 9.0. Uncorrected *p*-distances were calculated using PAUP* 4.0 (David Swofford, Sinauer Associates, Sunderland, Massachusetts).

The different regions of the target sequence (3'18S, ITS-1, 5.8S, ITS-2 and 5'28S) were identified in the alignments of each individual by comparing with the best match sequence available in the NCBI database (Table 2). The sequence identified under Accession no. AJ705046 (*Pseudaxinella reticulata* Nie, 2005 unpublished) was used in the alignment of individuals of *Phakellia* sp. and *Axinella aurensis*. AF340018 (*Astrosclera willeyana* Wörheide et al., 2000) was used in the alignment of *Acanthella cavernosa*.

Average uncorrected *p*-distance values were calculated for each individual and each region of the amplified sequence to assess the degree of IGV.

Neighbour-joining distance trees were constructed using PAUP* 4 to assess overall sequence similarity within individuals, between individuals of the same species and among the selected species.

RESULTS

Sequences for a region including the 3'end of 18S rDNA, ITS-1, 5.8S rDNA , ITS-2 and the 5'end of 28S rDNA of six clones and two individuals of the species *Phakellia* sp., *Axinella aruensis* and *Acanthella cavernosa* were obtained in this study.

The sequence length of the studied individuals (Table 3) varied on average from 848 to 897 base pairs (bp). The approximate length of the internal spacers and the 5.8S rDNA was 281–308 bp (ITS-1), 147–194 bp (5.8S) and 151–176 bp (ITS-2).

Single nucleotide differences among the sequenced clones and polymorphic sites were found in all the individuals studied. The distribution of informative polymorphic sites (i.e. excluding invariable, single nucleotide differences and gapped positions) for each species and individuals is presented in Figure 1. A total of 77 polymorphic positions through the length of the sequences were detected in individuals of

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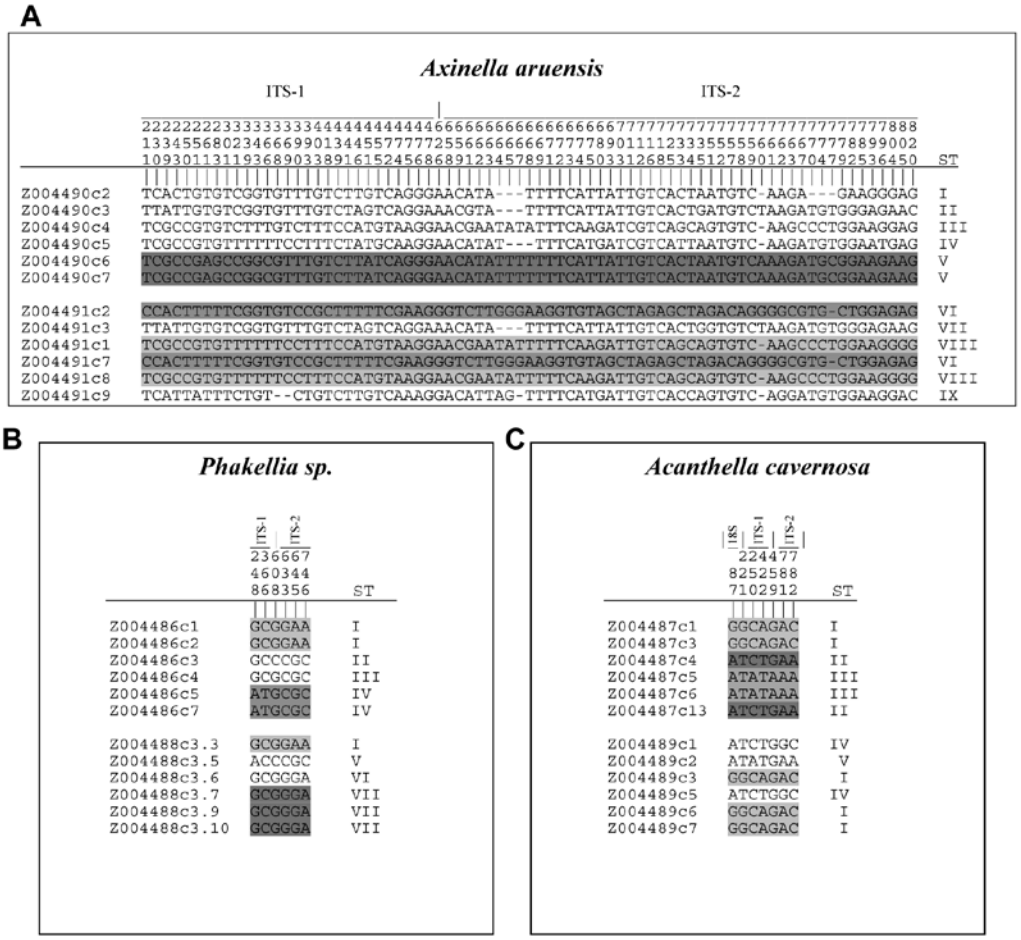

Figure 1. (A–C) Distribution of informative polymorphic sites in the sequenced clones of the studied species and individuals. Each alignment was compressed by deleting constant characters and uninformative positions (single nucleotide differences and gapped positions). Nucleotide position in the full-length alignment is indicated on top. Identical sequences are shaded with the same grey scale. Different sequences (ST, referred to in the text as sequence types) are identified with roman numerals.

Table 4. *Maximum and average (in parentheses)* p*-distances observed in the internal spacers of rDNA of selected sponges (including those investigated in this study) and corals.*

	ITS-1	ITS-2	Reference
Porifera (Demospongiae)			
Phakellia sp.			
Z004486	0.01068 (0.00498)	0.02125 (0.01053)	This study
Z004488	0.01650 (0.00550)	0.02533 (0.00881)	This study
Axinella aruensis			
Z004490	0.06091 (0.04039	0.11124 (0.06920)	This study
Z004491	0.08954 (0.06638)	**0.29152** (0.17371)	This study
Acanthella cavernosa			
Z004487	0.02883 (0.01487)	0.01765 (0.00902)	This study
Z004489	0.01764 (0.00893)	0.01136 (0.00492)	This study
Placospongia sp. (Baja, Mexico)	0.01885 (n.a.)	0.01563 (n.a.)	(Nichols & Barnes, 2005; Wörheide et al., 2004)
Prosuberites '*laughlini*' (UCMPWC525-Pacific)	0.0669 (n.a.)	0.04348 (n.a.)	(Wörheide et al., 2004)
Porifera (Hexactinellida)			
Aphrocallistes vastus	0.09113 (n.a.)	0.05532 (n.a.)	(Wörheide et al., 2004)
Cnidaria			
Acropora cervicornis (cer_Ja4 Caribbean)	0.114 (0.034)	0.051 (0.023)	(Vollmer & Palumbi, 2004)
Acropora aspera	~0.5 (n.a.)	n.a.	(in Van Oppen et al., 2000)

n.a., data not available; maximum value in bold.

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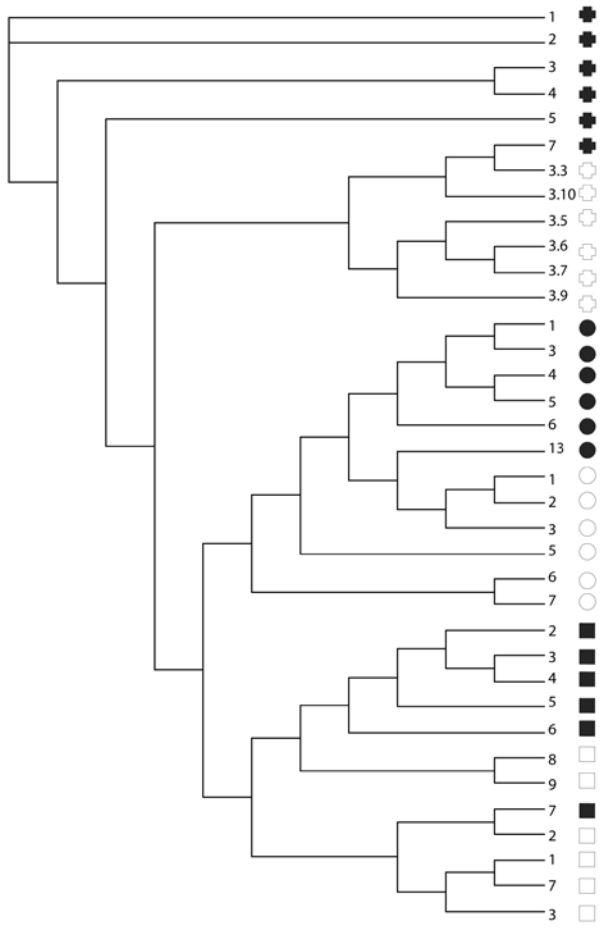

Figure 2. Neighbour-joining tree constructed using the alignment of full-length sequences of all clones from the studied species. Individuals are represented with different symbols next to the clone number. *Phakellia* sp., Z4486 ●, Z4488 ○; *Acanthella cavernosa*, Z4487 ●, Z4489 ○; *Axinella aruensis*, Z4490 ■, Z4491 □.

Axinella aruensis. The number of variable sites observed in the other two species was considerably less (six in *Phakellia* sp. and seven in *Acanthella cavernosa*). Most of these sites were observed in the internal spacers of the sequenced region with ITS-2 showing in general, more polymorphic sites than ITS-1. Variable sites found in the 18S, 5.8S and 28S regions of the majority of the sequences were not informative. Only one informative variable position was observed in the 18S region of *A. cavernosa*.

Excluding uninformative positions, the number of non-identical sequences (referred to as sequence types herein), varied within individuals, among individuals and among species (Figure 1). Three to five sequence types were found in any single individual with the maximum number found in one individual (i.e. Z004490) of *Axinella aruensis*. In two species (*Phakellia* sp. and *Acanthella cavernosa*) the same sequence type (i.e. sequence identified as I in both species, see Figure 1B,C) was detected in different individuals.

Intragenomic variation expressed as uncorrected *p*-distances and calculated from pairwise comparisons of the full-length sequences ranged in average from 0.00533 to 0.05331 (Table 3) with the maximum *p*-distance found in the two individuals of *Axinella aruensis*. Individuals of *Phakellia* sp. and *Acanthella cavernosa* showed similar levels of IGV. Maximum *p*-distances (Table 4) ranging from 1% to a maximum of 29% were detected in the ITS-2 of the two axinellid species. *Acanthella cavernosa* showed slightly higher values of IGV in the ITS-1 region instead. Very low values of IGV were observed in the 18S, 5.8S and 28S regions included in the sequences.

Neighbour-joining trees for each species were constructed using either full-length sequences, or including just the ITS spacers. Only the neighbour-joining tree constructed with the clones of all the studied individuals and the entire sequence is presented here (Figure 2). Slightly different topologies in the grouping of the clones within individuals were found among the constructed trees but more importantly, the sequenced clones did not cluster together within the individuals of each species. Individuals of each species, however, were clustered together, indicating that there is structure in the grouping at the intra-specific level.

DISCUSSION

In this study we investigated the degree of intragenomic variation of the internal spacers of the rDNA in three species of the order Halichondrida to determine whether or not these markers include levels of intra-individual polymorphisms that could confound phylogenetic/phylogeographical analyses of this sponge group.

The length of the internal spacers reported here is within the range found for other sponge taxa: 216–408 bp for ITS-1 and 91–487 bp for ITS-2 (Duran et al., 2004a; Wörheide et al., 2004) and similar to other species of Halichondrida (e.g. *Axinella corrugata* Lopez et al., 2002, *Pseudaxinella reticulata*, AJ705046; Nee, unpublished).

The IGV was observed in all individuals of the three selected species to different degrees, with *Axinella aruensis* showing the maximum number of sequence types and IGV values. At least three different sequence types were detected in any single individual of the selected taxa. The number of possible variants within any single population is unknown and could not be assessed within the scope of this study; but considering that three out of six sequences are different within any individual it can be concluded that ITS spacers of the selected species include paralogous copies that have not been homogenized to the same sequence type by concerted evolution and therefore these gene regions cannot be treated as a single loci. The results also suggest that in these species the rate of concerted evolution might be slower than the rate of variation generated by processes such as hybridization, polyploidism, chromosome segment duplication and non-homologous gene recombination (Alvarez & Wendel, 2003). Further investigations will be required to determine which of these mechanisms generate variation at the genomic level in halichondrid species.

The levels of IGV detected in both spacers of the two axinellid species were similar but there was a slightly greater variation in ITS-2. In other studied sponges (Table 4) the maximum variability, up to 7% and 9%, is observed in the ITS-1 of the demosponge *Prosuberites laughlini* and the hexactinellid *Aphrocallistes vastus* respectively. According to Coleman (2003), ITS-2 contains conserved regions with sufficient phylogenetic signal for evolutionary comparisons of disparate groups of eukaryotes. This might not be the case

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for some of the sponges studied here given that the maximum variability found within some individuals was found in the ITS-2. The ITS-2 secondary structure of some eukaryotes, and some studied sponges, display universal features, with four helices, each with a stem and a loop. Such structure is maintained across groups by the presence of compensatory base changes (Coleman, 2003; Schmitt et al., 2005). Individuals with significant levels of intragenomic variation had variant nucleotide positions in regions where the secondary structure remains unpaired (Coleman, 2003). The comparison of the secondary structure of ITS-2 in axinellid sponges is therefore an issue that needs investigation and will provide insights to understand the variability observed here and to determine how the secondary structure of this transcript can be utilized for evolutionary comparisons with other sponges or eukaryotes.

It is intended in future work to elucidate the secondary structures of the obtained sequences not only to be utilized in phylogenetic analyses but to confirm that the detected polymorphisms are not the result of PCR and cloning artefacts, an issue that cannot be discarded as possible and that could explain sequence variation at similarity levels below 3% (Speksnijder et al., 2001; Wörheide et al., 2004 and references within).

In this study a maximum variation of 29% was recorded in the ITS-2 of *Axinella aruensis*. The overall average IGV in the full-length sequences of this species was 2–5% (Table 3), which is higher than the sequence divergence reported by Lopez et al. (2002) among individuals of the Caribbean species *A. corrugata* (<1%). The IGV reported here for *A. aruensis* is, to our knowledge, the highest recorded within Porifera. Values of similar magnitude have been detected in species of the coral genus *Acropora* (see Table 4). These levels of intra-individual variation, which are considered high within the literature, correspond in most cases to hybrid species (Van Oppen et al., 2000 and references therein; Coleman, 2003; Vollmer & Palumbi, 2004) and suggest that the IGV found in *Axinella aruensis* could be attributed to either ancestral or recent hybridization events.

Putative hybridization among Mediterranean species of *Axinella* was reported by Solé-Cava et al. (1991) after the discovery of a 'morph' with morphological characters overlapping between the species *A. damicornis* and *A. verrucosa*. The study by these authors reveals, however, that genetic differences evaluated by allozyme electrophoresis and expressed as genetic identity, were sufficient to consider the intermediate 'morph' as a different species, and highlighted the failure of traditional morphological methods to identify sponge species. No indication of hybridization was reported by Lopez et al. (2002) in *A. corrugata*.

Only thorough population genetic studies using a representative number of individuals from sympatric species and populations, multilocus genetic data from nuclear and mitochondrial markers (Erpenbeck et al., 2005c), and studies on the reproductive biology of the species will be able to answer whether or not *Axinella aruensis* is part of the sponge hybridization system. Current morphological studies by one of us (B.A.) will help to identify variability within this species that could be correlated with hybrid morphotypes if any are discovered.

The data obtained here suggest that ribosomal spacers might be suitable for phylogenetic analyses at, and above, genus level within the order Halichondrida. The variation detected within individuals of the screened species, however, might affect relationships at the genus level, despite species-specific clusters being found in the analysed data. Inclusion of additional species in similar analyses should be considered to confirm that ITS markers are appropriate for phylogenetic studies at the intragenic level. Equally, population studies at larger (among geographical areas) and finer (within a locality) scales could be confounded by the presence of IGV as indicated here, by the lack of structure in the grouping of the sequenced clones, and by the presence of identical sequence types in different individuals. Similar results are reported for other sponges with relatively high levels of IGV such as *Placospongia* sp. (Nichols & Barnes, 2005) and *Prosuberites laughlini* (Wörheide et al., 2004) suggesting that, the rDNA spacers are not appropriate markers for fine-scale population studies that include species with levels of IGV greater than 1% (Table 3).

In conclusion, sequence analyses of individuals and subclones (5–10 clones as recommended by Vollmer & Palumbi, 2004) from sympatric species, especially those included in *Axinella*, and populations from different geographical areas, are necessary for any phylogenetic analyses at lower levels of classification and phylogeographic studies of Halichondrida. As suggested by other authors (Coleman, 2003; Wörheide et al., 2004; Schmitt et al., 2005) secondary structure constraints should be used to align homologous sites of ITS-2 sequences of the taxa under consideration, to check for PCR and sequencing artefacts, recognition of pseudogenes and hybridization events. Additionally, nuclear and mitochondrial markers should be included as independent sources of data to complement lower level phylogenetic and biogeographical studies based on ITS sequences.

Finally, this study raises the possibility that at least some species of *Axinella* might have resulted from hybridization events that are worth investigating and which may explain the polyphyletic and enigmatic relationships recognized within this genus (Alvarez et al., 2000; Erpenbeck et al., 2005a, 2006).

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