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New polymorphic mitochondrial markers for sponge phylogeography

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Phylogeography and population genetic studies in the Porifera have been limited by the lack of available polymorphic DNA markers. In this paper, we tested four new mitochondrial markers in nine demosponge species from a wide taxonomic range: partial sequences of the ATP synthase 6 (ATP6) and the cytochrome oxidase 2 (CO2) genes and two spacers: one located between ATP6 and CO2 and the other between the NADH dehydrogenase subunit 5 (ND5) and the small subunit ribosomal RNA (rns) genes. The new markers presented levels of nucleotide diversity up to 2.4 times higher ($\pi = 0.015$ for CO2) than those observed for the most commonly used mitochondrial marker in sponges, the cytochrome oxidase 1 gene ($\pi = 0.006$), making them suitable for alpha-level systematics, phylogeography and population genetics studies.

Keywords: DNA markers, Porifera, population genetics

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INTRODUCTION

Sponge taxonomy is based primarily on the characteristic features of the skeleton, particularly the shape and size of its constitutive elements (Boury-Esnault, 2006), whose levels of inter- and intra-specific variation are often hard to discriminate, making them prone to large subjective interpretations by taxonomists (Hooper *et al.*, 1991). The paucity of diagnostic characteristics for taxon delimitation in sponges makes their systematics very complex and often conservative (Hooper *et al.*, 1991; Klautau *et al.*, 1999). Although many studies apply different approaches to complement morphological information, we are still very far from knowing the true number of extant sponge species (Boury-Esnault, 2006). Furthermore, the difficulty in establishing homologies among skeletal elements and their organization hinders the comprehension of evolutionary relationships among sponge taxa, especially among sibling and cryptic species (Boury-Esnault *et al.*, 1994; Klautau *et al.*, 1994; Wulff, 2006).

For many marine invertebrates, like crustaceans (Groeneveld *et al.*, 2007; Palero *et al.*, 2008), molluscs (Imron *et al.*, 2007; Baker *et al.*, 2008; Polson *et al.*, 2009), annelids (Wiklund *et al.*, 2009) and echinoderms (Muths *et al.*, 2009; Owen *et al.*, 2009) mitochondrial DNA (mtDNA) has proved to be an excellent marker for studies of population genetic structure, dispersal and historical biogeography (Avise, 1986). Indeed, mtDNA presents a number of theoretical and practical advantages over nuclear DNA for phylogeography, since, in addition to its high rate of evolution, it is maternally inherited avoiding, thus, problems related to recombination, and has coalescent times three-times shorter than those of nuclear markers (Hare, 2001).

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However, for basal metazoans, such as sponges and cnidarians, it has been shown that the evolutionary rate of mtDNA can be 10 to 20 times lower than that of the Bilateria (Shearer *et al.*, 2002; van Oppen *et al.*, 2002; Duran *et al.*, 2004a; Wörheide, 2006). The reported lack of intra-specific variation of mtDNA in sponges and cnidarians led van Oppen and colleagues (2002) to advise against its use for studies at low taxonomic levels. Most studies that showed the conservativeness of mtDNA in sponges have used part of the cytochrome oxidase 1 (CO1) gene (Duran *et al.*, 2004a; Wörheide, 2006). The conservative features of the CO1 gene in sponges can be evidenced by its use at higher taxonomic level studies, such as relationships among genera (Heim *et al.*, 2007), families (Erpenbeck *et al.*, 2002; Addis & Peterson, 2005; Itskovich *et al.*, 2006) and even orders (Nichols, 2005; Erpenbeck *et al.*, 2007). The I3M11 partition has been suggested as an alternative to the more commonly used 5′ partition CO1, because it has a lower transition/transversion ratio in the third position of codons (Erpenbeck *et al.*, 2006). Levels of nucleotide diversity (π) for this marker in 7 populations of *Xestospongia muta* (4 haplotypes; $\pi = 0.00386$) were higher than those found for the 5′ partition ($\pi = 0.00058$) (López-Legentil & Pawlik, 2009) widely applied in metazoan population studies. Sequences of the mitochondrial NADH dehydrogenase subunit 5 (ND5) have also been analysed for population studies of the sponge *Hymeniacidon synapium*, but only two haplotypes were found in 18 localities around Japan and South Korea (Hoshino *et al.*, 2008).

Due to the paucity of molecular markers at lower taxonomic levels, most alpha taxonomy, population genetics and phylogeography studies of sponges have been limited to allozymes, which present high levels of polymorphism (Solé-Cava & Thorpe, 1989). Allozymes are ubiquitous and codominant, making them powerful tools for the detection of reproductive isolation in sympatry and, hence, very useful in alpha-taxonomy (Davis *et al.*, 1996; Solé-Cava & Boury-Esnault, 1999). However, the study of sponge allozymes is limited by

the requirement of fresh or frozen samples (Boury-Esnault & Solé-Cava, 2004). Ribosomal spacers have also been used (Wörheide *et al.*, 2002, 2008), but their utility is hampered in many sponge species by high intragenomic variability (Wörheide *et al.*, 2004; Alvarez *et al.*, 2007). Microsatellites are good markers for sponge population genetics and molecular ecology (Duran *et al.*, 2004b; Blanquer *et al.*, 2009; Noyer *et al.*, 2009), but their low taxonomic ubiquity means that species-specific markers must be developed *de novo* for each species. Thus, there is currently much interest in the development of new polymorphic mitochondrial markers for sponge systematics, phylogeography and population genetics (Erpenbeck *et al.*, 2007; Hoshino *et al.*, 2008). In this paper, we describe new primer systems for the amplification and sequencing of four new mitochondrial polymorphic markers in nine species from a wide taxonomic range of demosponges.

MATERIALS AND METHODS

The target fragments were chosen after aligning all available sponge mitochondrial genomes using CLUSTALW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Larkin *et al.*, 2007). Based on the aligned mitochondrial DNA sequences, four pairs of primers were designed in conserved regions that flanked variable regions (Table 1). Two of those markers include genes and intergenic regions (Table 2), whereas the other two markers amplify partial sequences of the ATP synthase 6 (ATP6) and the cytochrome oxidase 2 (CO2) genes. To compare the variability levels of the four new markers, part of the CO1 gene was amplified using a universal pair of primers (Folmer *et al.*, 1994).

The primers were tested in nine sponge species that encompass six orders of the class Demospongiae (Table 1). Whenever possible, samples from two populations per species were used to determine the level of variability for population genetics studies. This study also includes two species of the genera *Placospongia* and *Chondrosia*, to estimate levels of inter-specific variability.

The analysed sponge samples cover a wide range of sponge orders and geographical locations. *Chondrosia* aff. *reniformis* were collected in the south-west Atlantic (Arraial do Cabo—south-east Brazil and Guarajuba—north-east Brazil) and in the north-west Atlantic (Bermuda); *Chondrosia reniformis* are from the western Mediterranean Sea (Marseille, France) *Placospongia* aff. *carinata* are from the south-west Atlantic (Calheta and Ponta dos Seixas, both in north-east Brazil) and the Atlantic (Bocas del Toro) and Pacific (Galleta) coasts of Panama; *Placospongia* aff. *melobesioides* are from the south-west Atlantic (Guarajuba and Calheta, both in north-east Brazil); *Cliona delitrix* are from the north-west and south-west Atlantic (Lee Stocking Island, the Bahamas and the Abrolhos reefs—north-east Brazil); *Cinachyrella* sp. are from Atlantic Panama (Bocas del Toro) and the south-west Atlantic (Ponta dos Seixas and Pontal do Maracaípe—north-east Brazil); *Aplysina fulva* are from Atlantic Panama (Bocas del Toro); *Hymeniacidon heliophila* are from the south-west Atlantic (Rio de Janeiro—south-east Brazil); and *Amphimedon erina* are from the south-west Atlantic (Ponta dos Seixas—north-east Brazil).

The genomic DNA was obtained from sponge pieces smaller than 0.5 cm^3 by lysing the tissue overnight in a guanidine solution (4 M guanidine hydrochloride, 50 mM Tris HCl

Table 1. Primer sequences and annealing temperature of the markers and sponge species tested. * In the case of *Aplysina fulva* the reverse primer used was CO2aplyR: ACTAAMACTCGCACTTGGGTTTG.

Name	Primer sequence (5′–3′)	PCR annealing temperature						
		Aplysina fulva	*Amphimedon erina*	*Chondrosia* aff. *reniformis/reniformis*	*Cinachyrella* sp.	*Cliona delitrix*	*Hymeniacidon heliophila*	*Placospongia* aff. *carinata/melobesioides*
Cytochrome oxidase 1 (CO1) (Folmer *et al.*, 1994)								
CO1F	GGTCAACAAATCATAAAGATATTGG							
CO1R	TAAACTTCAGGGTGACCAAAAAATCA	48°C	–	48°C	48°C	48°C	48°C	48°C
ATP synthase 6 (ATP6)								
ATP6porF	GTAGTCCAGGATAATTTAGG							
ATP6porR	GTTAATAGACAAAATACATAAGCCTG	46°C	34°C	54–48°C	53–48°C	53–48°C	48 °C	48°C
Cytochrome oxidase 2 (CO2)								
CO2F	TTTTTCACGATCAGATTATGTTTA							
CO2R	ATACTCGCACTGAGTTTGAATAGG	40°C*	34°C	54–48°C	54–42°C	50–44°C	54–42 °C	51–45°C
Spacer region 1 (SP1)								
CO2Fc	TGTKGCGCAAATCATTCWTTTATGC							
ATP6R	TGATCAAAATAWGCTGCTAACAT	56–44°C	34°C	51°C	44–34°C	50–45°C	44–34°C	53–48°C
Spacer region 2 (SP2)								
ND5F	GTGTTCAACTATGCTTTAATWATGAT							
rnsR	CGTACTTTCATACATTGYAC	44–34°C	–	49°C	58–44°C	46–40°C	44–34°C	53–48°C

Table 2. Order of the genes and spacer regions from the analysed species. For comparison we present literature data from sponges from the same orders. Inside the parentheses are the numbers of base pairs (bp). References: 1, Lavrov *et al.* (2008); 2, Wang & Lavrov (2008); 3, Watkins & Beckenbach (1999); 4, Lavrov *et al.* (2005); 5, Lukic-Bilela *et al.* (2008); 6, Lavrov & Lang (2005).

Species	Order	SP1 (CO2–ATP6)	SP2 (ND5–rns)	Reference
Aplysina fulva	Verongida	CO2-**SP(114)**-trnK-ATP8-ATP6	ND5-**SP(8)**-trnA-**SP(41)**-trnM-**SP(8)**-trnF-rns	1
Aplysina fulva	Verongida	CO2-**SP(114)**-trnK-ATP8-ATP6	ND5-**SP(8)**-trnA-**SP(41)**-trnM-**SP(8)**-trnF-rns	This paper
Amphimedon compressa	Haplosclerida	CO2-**SP(30)**-trnK-ATP8-ATP6	ND5-trnA-**SP(12)**-CO2-**SP(30)**-trnK-ATP8-ATP6, etc	2
Amphimedon queenslandica	Haplosclerida	CO2-**SP(76)**-trnK-ATP8-**SP(14)**-ATP6	ND5-**SP(11)**-trnA-**SP(20)**-CO2-**SP(76)**-trnK, etc	
Amphimedon erina	Haplosclerida	CO2-**SP(29)**-trnK- ATP8-ATP6	Not sequenced	This paper
Chondrilla aff. *nucula*	Chondrosida	CO2-**SP(65)**-trnK-**SP(23)**-ATP8-ATP6	ND5-**SP(20)**-trnA-CO2-trnK-ATP8-ATP6-trnI, etc	1
Chondrosia aff. *reniformis*	Chondrosida	CO2-trnK-**SP(53)**-ATP8-ATP6	ND5-**SP(270)**-rns	This paper
Cinachyrella kuekenthali	Spirophorida	CO2-trnK-ATP8-ATP6	ND5-trnA-**SP(16)**-trnF-rns	1
Tetilla sp.	Spirophorida	CO2-**SP(14)**-trnL-ATP8-ATP6	Not sequenced	3
Cinachyrella sp.	Spirophorida	CO2-trnK-ATP8-ATP6	ND5-trnA-**SP(15)**-trnF-rns	This paper
Tethya actinia	Hadromerida	CO2-**SP(38)**-trnL-**SP(320)**-trnK-ATP8-ATP6	ND5-**SP(29)**-trnA-trnM-**SP(121)**-trnF-rns	4
Suberites domuncula	Hadromerida	CO2-**SP(404)**-trnK-ATP8-**SP(297)**-ATP6	ND5-**SP(263)**-trnA-**SP(20)**-trnM-**SP(383)**-trnF-rns	5
Cliona delitrix	Hadromerida	CO2-**SP(80)**-trnK-ATP8-**SP(4)**-ATP6	ND5-**SP(28)**-trnA-**SP(16)**-trnM-**SP(122)**-trnF-rns	This paper
Placospongia aff. *melobesioides*	Hadromerida	CO2-**SP(18)**-trnL-**SP(235)**-trnK-ATP8-**SP(9)**-ATP6	ND5-**SP(12)**-trnA-**SP(19)**-trnM-**SP(130)**-trnF-rns	This paper
Placospongia aff. *Carinata*	Hadromerida	CO2-**SP(12)**-trnL-**SP(235)**-trnK-ATP8-**SP(9)**-ATP6	ND5-**SP(12)**-trnA-**SP(5)**-trnM-**SP(141)**-trnF-rns	This paper
Axinella corrugata	Halichondrida	CO2-**SP(248)**-trnC-**SP(105)**-trnN-**SP(34)**-trnK, etc	ND5-**SP(143)**-trnL-**SP(257)**-trnF-rns	6
Ptilocaulis walpersi	Halichondrida	CO2-**SP(89)**-trnK-ATP8-ATP6	ND5-trnA-**SP(312)**-trnD-trnM-trnF-rns	1
Topsentia ophiraphidites	Halichondrida	CO2-**SP(67)**-trnK-ATP8-ATP7	ND5-trnA-**SP(19)**-trnM-**SP(57)**-ND4-**SP(40)**-trnH, etc	1
Hymeniacidon heliophila	Halichondrida	CO2-**SP(236)**-trnK-ATP8-**SP(253)**-ATP6	ND5-**SP(267)**-trnA-**SP(5)**-trnM-**SP(223)**-trnF-rns	This paper

pH 8.0, 0.05 M EDTA, 0.5% sodium-N′-lauroylsarcosine and 1% ß-mercaptoethanol) (Lôbo-Hajdu *et al.*, 2004) with proteinase K at 55°C, followed by a phenol–chloroform extraction. Amplification reactions were performed in a 15 μl volume, containing 1 μl (90 ng) of genomic DNA, 1.5 mM $MgCl_2$, 200 μM of dNTP mix, 0.5 μM of each primer and 1 U of Taq DNA polymerase. Cycling conditions for CO1 started with an initial cycle at 94°C for 3 minutes, followed by 35 cycles of denaturing at 93°C for 1 minute, annealing at 48°C for 1 minute and extension at 72°C for 1 minute, and one final extension step at 72°C for 4 minutes. For all other DNA markers, the polymerase chain reaction (PCR) conditions followed cycles similar to those used for CO1, however, a touchdown PCR was performed in which the annealing temperature was decreased by 1°C during each of the first 6 cycles, followed by 29 cycles with the lower annealing temperature, as shown in Table 1.

The PCR products were visualized on 1.5% agarose gels, purified using ExoSap-IT (USB Corporation, Cleveland, OH, USA) or QIAquick PCR Purification Kits (QIAGEN) and sequenced for both the forward and reverse strands using ABI Big Dye chemistry on an ABI 3130 DNA sequencer (Applied Biosystems, Foster City, CA, USA). All sequences were edited using the SEQMANII software program (DNASTAR, Inc.) and aligned in ClustalX with the Mega 4 software (Tamura *et al.*, 2007). The aligned sequences were meticulously inspected and edited when necessary. Nucleotide (π) and haplotype (h) diversities and Jukes–Cantor DNA divergence between congeneric species of *Placospongia* and *Chondrosia* were estimated using DnaSP 5.10 (Librado & Rozas, 2009). Hairpin-forming elements have been reported within mitochondrial spacers of some demosponges, and their repetitive nature makes them a potential source of noise in evolutionary analyses (Erpenbeck *et al.*, 2009). Thus, the presence of repetitive sequences in intergenic regions was checked using the Tandem Repeat Finder 4.00 program (Benson, 1999).

RESULTS

Optimal temperatures for PCR amplification were established for the four new pairs of primers (Table 1). The SP2 primer system did not result in the amplification of any fragment in *Amphimedon erina*, but that was expected, considering that Haplosclerida present a gene order different from that of the other sponges tested (Wang & Lavrov, 2008).

The highest value of nucleotide diversity was 0.042 in *Cinachyrella* sp. for the SP2 marker, and the highest value of haplotype diversity was 0.933 in *Chondrosia* aff. *reniformis* with the SP1 marker (Table 3). Interestingly, no variation was observed in any of the markers of *Aplysina fulva*, *Hymeniacidon heliophila* or *Chondrosia reniformis* (Table 3).

The new markers were more polymorphic than CO1 in all species where any polymorphism was detected (Table 3). In some extreme cases, like CO2 in *Placospongia* aff. *melobesioides*, the new marker presented a nucleotide diversity six times higher than that observed with CO1 (Table 3).

Levels of gene divergence between the two species of *Placospongia* and *Chondrosia* were also higher for the new markers than for CO1 (mean Jukes–Cantor $p = 0.0982$ against $p = 0.0149$ for CO1 for *Placospongia* and mean Jukes–Cantor $p = 0.0476$ against $p = 0.0431$ for CO1 for *Chondrosia*). The comparisons between the species of *Placospongia* resulted in mean values of $p = 0.020$ (CO2); 0.065 (ATP6); 0.058 (SP1); 0.066 (SP2) and, for *Chondrosia* spp., mean $p = 0.056$ (CO2); 0.039 (ATP6); 0.059 (SP1); 0.036 (SP2).

Finally, repetitive hairpin-forming motifs were observed only in *Chondrosia* aff. *reniformis*, which presented two small (twelve-nucleotide stems) hairpins in the SP2 fragment.

DISCUSSION

This study describes four new polymorphic mitochondrial markers, which will provide evolutionary ecologists with the needed tools for studies of sponge alpha taxonomy, phylogeography and population genetics.

Additionally, this study contradicts previous suggestions on the high conservativeness of the sponge mitochondrial genome (van Oppen *et al.*, 2002) and, as predicted by some researchers (Wörheide *et al.*, 2005, Wang & Lavrov, 2008), confirms that the sponge mtDNA contains regions variable enough for analyses at the population and alpha taxonomy levels. For example, the new mitochondrial markers were over two times more divergent than CO1 between congeneric species of *Placospongia*. Also, the observed relationship between inter- and intra-specific differentiation was about 2.3 times higher for the new markers than in CO1 (mean Jukes–Cantor $p_{inter}/p_{intra} = 45.1$ and 18.8, respectively), which indicates that they will be less restrained than CO1 (Shearer *et al.*, 2002) for studies of alpha-level sponge systematics.

In a few cases, CO1 has been shown to be useful for population genetics and detection of cryptic species, like in the study of *Callyspongia vaginalis* along the Florida reef tract (DeBiasse *et al.*, 2010) and in the finding of cryptic species of *Cliona celata* (Xavier *et al.*, 2010). In both cases, levels of nucleotide diversity were similar to those found with the new markers (π from 0.001 to 0.042; Table 3).

The CO2 fragment was not amplified in *Aplysina fulva* (Table 3), even after exhaustive tests with different annealing temperatures and PCR-reagent concentrations. An analysis of the published sequence of the CO2 gene in *Aplysina fulva* showed that the reverse primer annealing site, which is conserved across other sponge orders, has five nucleotide differences which were probably responsible for the failed amplifications. Thus, we designed a new reverse primer which produced two sequences of CO2 with approximately 800 and 400 base pairs. The latter was used in the analyses because it has the expected size and produced the most reliable sequences. The same difficulty in amplification was observed for CO1, which failed to amplify in *Amphimedon erina* over a wide range of experimental conditions (Table 1). Since CO1 was not the aim of this work, we did not further pursue this matter.

The lack of sequence variation in any of the analysed markers in *Chondrosia reniformis*, *Hymeniacidon heliophila* and in *Aplysina fulva* is more likely the result of the analysis of clone-mates, since those three species are known to reproduce asexually, sometimes quite extensively (Stone, 1970; Wulff, 1991; Tsurumi & Reiswig, 1997; Bavestrello *et al.*, 1998). Although intra-population variability was observed in all other species analysed, it is noteworthy that for these three species we had samples from only one locality. It

Table 3. Haplotype (h) and nucleotide (π) diversity of each species for each marker. The number of sequences and populations used in the analyses are presented below the diversity indices. Mean diversity values exclude the potentially clonal populations of *Chondrosia* aff. *reniformis*, *Aplysina fulva* and *Hymeniacidon heliophila*. The ratio between variabilities of the new markers and those of CO1 were calculated over the loci that were sequenced for each species. "–", did not amplify; seqs, sequences; pop, population.

Species	CO1		ATP6		CO2		CO2/ATP6 (SP1)		ND5/rns (SP2)	
	h	π	h	π	h	π	h	π	h	π
Aplysina fulva	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	(5 seqs -1 pop)		(5 seqs - 1 pop)		(5 seqs - 1 pop)		(5 seqs - 1 pop)		(3 seqs - 1 pop)	
Amphimedon erina	–	–	0.000	0.000	0.000	0.000	0.400	0.001	too long fragment	
	–		(3 seqs - 1 pop)		(3 seqs - 1 pop)		(5 seqs - 1 pop)			
Chondrosia aff. *reniformis*	0.712	0.002	0.000	0.000	0.667	0.004	0.714	0.003	0.250	0.001
	(12 seqs - 7 pop)		(5 seqs - 3 pop)		(4 seqs - 2 pop)		(7 seqs - 3 pop)		(8 seqs - 4 pop)	
Chondrosia reniformis	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	(4 seqs - 1 pop)		(2 seqs - 1 pop)		(3 seqs - 1 pop)		(3 seqs - 1 pop)		(3 seqs - 1 pop)	
Cinachyrella sp.	0.400	0.024	0.722	0.017	0.556	0.031	0.750	0.013	0.714	0.042
	(5 seqs - 3 pop)		(9 seqs - 3 pop)		(9 seqs - 3 pop)		(8 seqs - 3 pop)		(7 seqs - 3 pop)	
Cliona delitrix	0.639	0.001	0.556	0.001	0.333	0.001	0.714	0.002	0.600	0.002
	(9 seqs - 2 pop)		(10 seqs - 2 pop)		(6 seqs - 2 pop)		(7 seqs - 2 pop)		(5 seqs - 2 pop)	
Hymeniacidon heliophila	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	(10 seqs - 1 pop)		(10 seqs - 1 pop)		(6 seqs - 1 pop)		(6 seqs - 1 pop)		(8 seqs - 1 pop)	
Placospongia aff. *carinata*	0.167	0.001	0.495	0.001	0.564	0.003	0.868	0.005	0.000	0.000
	(12 seqs - 4 pop)		(14 seqs - 4 pop)		(13 seqs - 4 pop)		(14 seqs - 4 pop)		(15 seqs - 4 pop)	
Placospongia aff. *melobesioides*	0.524	0.001	0.250	0.001	0.667	0.006	0.000	0.000	0.000	0.000
	(7 seqs - 2 pop)		(8 seqs - 2 pop)		(3 seqs - 1 pop)		(7 seqs – 2 pop)		(9 seqs - 2 pop)	
Mean value	0.518	0.006	0.500	0.008	0.576	0.015	0.653	0.006	0.403	0.010
Marker/CO1			0.964	1.267	1.112	2.433	1.260	0.967	0.777	1.600

would be interesting to confirm the hypothesis of extensive clonality in these three species using hyper-variable markers, like microsatellites.

Recent data demonstrate that intergenic regions may have repetitive hairpin-forming elements that can lead to misleading phylogenetic signals due to independent origins and evolution (Erpenbeck *et al.*, 2009). No hairpin-forming repetitive sequences were found for the intergenic region SP1 in any of the analysed sponges. In *Chondrosia* aff. *reniformis*, two small (twelve-nucleotide stems) hairpins were found in SP2. The repeat was not found in any of the other tested species, including the congeneric *Chondrosia reniformis*. In spite of their low frequency, repetitive sequences should be searched for whenever intergenic spacers are analysed.

Applying a large array of molecular markers is desirable for phylogeography and molecular systematics analyses (Beheregaray, 2008). The four new markers described here amplified efficiently and were more variable than CO1 in the polymorphic sponges tested (Table 3). Therefore, they will be useful to complement the markers available for sponge studies. This will help in overcoming typical problems linked to the use of single markers for population genetics, such as the presence of pseudogenes and other sources of homoplasy. More importantly, the use of genes with different evolutionary rates will help to circumvent the pitfalls of using single-gene trees, which tell a limited story of the species, to draw general conclusions in systematics and phylogeography (Solé-Cava & Wörheide, 2007).

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