A novel biochemical method to distinguish cryptic species of *Chondrilla* (Chondrosida, Demospongiae) based on its sulfated polysaccharides

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Abstract: Sulfated polysaccharides from marine sponges are highly complex molecules with species specific composition. We now propose a novel biochemical method to distinguish cryptic species of *Chondrilla*, built on the analysis of the sulfated polysaccharides content. The major difference between the sulfated polysaccharides from *Chondrilla australiensis* and *Chondrilla nucula* is their sulfate content, which was enough to give different electrophoretic motilities on agarose gel. Additionally, the sulfated polysaccharides from the cryptic species *C. nucula*, *Chondrilla* sp. B, *Chondrilla* sp. E and *Chondrilla* sp. F also showed distinct electrophoretic motilities on agarose gel. This method allowed the distinction of two sympatric cryptic species of *Chondrilla* (sp. E and sp. F) found through allozymes by the presence of a diagnostic locus. Analysis of the sulfated polysaccharides has advantages over allozymes or DNA since it can be applied to specimens fixed either in ethanol, formaldehyde, frozen or dried.

Keywords: marine sponges, molecular systematics, glyconectins

Introduction

The cellular adhesion and recognition of marine sponges (Porifera) is mediated by proteoglycan-like molecules, also called aggregation factors (AF´s), spongicans or glyconectins (*e.g.* Fernàndez-Busquets and Burger 2003, Guerardel *et al.* 2004, Misevic *et al.* 2004). These proteoglycan-like molecules are composed of a protein core attached to several sulfated polysaccharide units (Humphreys *et al.* 1977, Jarchow *et al.* 2000). The sulfated polysaccharide units of glyconectins are responsible for the cell-cell recognition and adhesion in sponges (Bucior and Burger 2004). The interaction between the sulfated polysaccharides of adjacent sponge cells is calcium dependent and a highly species specific event (Spillmann and Burger 1996, Bucior and Burger 2004, Misevic *et al.* 2004).

The species specific interaction of the sulfated polysaccharides from glyconectins was demonstrated by the selective and homophilic aggregation of beads coated with sulfated polysaccharides from different sponges (Popescu and Misevic 1997, Misevic *et al.* 2004). Another evidence for the species specificity of sulfated polysaccharides from Porifera species is their chemical and structural diversity (Zierer and Mourão 2000, Guerardel *et al*. 2004). These sulfated polysaccharides are highly complex and all the species previously studied showed polymers with different structures and/or sugar and sulfate content (Table 1).

The taxonomy of sponges is an unsolved problem due to the low numbers of usable morphological characters to discriminate species (Solé-Cava *et al.* 1991, Solé-Cava 1994). Due to the lack of consistent morphological traits, many species of sponges are considered cosmopolitan (Solé-Cava *et al.* 1991). However, recent studies using molecular markers, such as allozymes and DNA sequences, showed that many species considered cosmopolitan were actually complexes of cryptic species (e.g., Solé-Cava and Thorpe 1986, Boury-Esnault *et al.* 1992, Muricy *et al.* 1996, Klautau *et al.*1999, Lazoski *et al.* 2001, Loukaci *et al.* 2004, Usher *et al.* 2004). Although analysis of allozymes seems to have enough resolution to distinguish cryptic species, the use of this methodology is impounded due to the need of fresh or frozen samples (Wörheide *et al.* 2004). In addition, comparisons among allozymes and other currently available molecular markers in detecting cryptic species of sponges have yield conflicting results (Zilberberg 2006). Therefore, there is a great need to find novel markers for the detection of cryptic species of sponges.

Table 1: Chemical differences among the sulfated polysaccharides from marine sponges.

Species	Sulfated polysaccharide	Reference
Aplysina fulva	HexUA, Glu, (sulfated)	Zierer and Mourão 2000
Chondrilla nucula	HexUA, Ara, Gal, Fuc, (sulfated)	Zierer and Mourão 2000
Cliona celata	Sulfated HexNac, Ara, Fuc	Guerardel *et al.* 2004
Dysidea robusta	HexUA, Ara, Gal, Fuc 4-*O*-sulfated	Zierer and Mourão 2000
Halichondria panicea	Gal Py(4,6), Fuc, GlcNac *N*-sulfated	Guerardel *et al.* 2004
Hymeniacidon heliophila	HexUA, Gal, Fuc, (sulfated)	Zierer and Mourão 2000
Microciona prolifera	Gal, Fuc, Gal Py (4,6), GlcNac *N*-sulfated	Guerardel *et al*. 2004
Myxilla rosacea	Glc 4,6-disulfated, Fuc 2,4-disulfated	Cimino *et al.* 2001
Ophlithaspongia tenius	HexUA, Glc, GlcNac *N*-sulfated	Parrish *et al.* 1991
Suberites ficus	HexUA, GlcNac, Fuc, Man, Gal (sulfated)	Bucior and Burger 2004

Chondrilla (Demospongiae: Chondrillidae) is a good model for the detection of cryptic species due to the large number of cryptic species that have been found along the Atlantic and Pacific Oceans (Klautau *et al*. 1999, Usher *et al*. 2004, Zilberberg 2006, Zilberberg *et al*. 2006). Usher *et al*. (2004) found, through DNA sequence analyses, two cryptic species of *Chondrilla australiensis* along the western coast of Australia. Similarly, along the Atlantic Ocean there are about six to eight cryptic species of *Chondrilla nucula*, which have been found through allozymes or DNA sequence analyses (Klautau *et al*. 1999, Zilberberg 2006, Zilberberg *et al.* 2006).

Based on the species specificity of the sulfated polysaccharides from sponges, we propose, here, a novel biochemical method to distinguish cryptic species within the genus *Chondrilla* through agarose gel electrophoresis of its sulfated polysaccharides. The chemical composition of the sulfated polysaccharides from the species *C. nucula* and *C. australiensis* were analyzed to evaluate the differences between sulfated polysaccharides from congeneric species. We also tested the efficiency of this methodology in detecting cryptic species of *Chondrilla* found through allozymes (Klautau *et al.* 1999, Zilberberg *et al.* 2006). Additionally, we tested the ability to analyze specimens fixed in different media, including formaldehyde, a preservative that makes the study of allozymes and DNA sequences unfeasible.

Material and methods

Sponge samples

To analyze the sulfated polysaccharides from cryptic species of *Chondrilla*, two specimens from each of five species were used. These species were: *Chondrilla australiensis* (Melbourne, Australia); *C. nucula* (Marseille, France); two cryptic species found in sympatry in the Bahamas (Lee Stocking Island), named *Chondrilla* sp. E and sp. F (Zilberberg *et al.* 2006); and *Chondrilla* sp. B (Klautau *et al*. 1999), one individual from Ubatuba (São Paulo, Brazil) and another from Búzios (Rio de Janeiro, Brazil). All these specimens were fixed in 70% ethanol. Additionally, to compare the efficiency of this methodology using different fixatives, four specimens of *Chondrilla* sp. B from Arraial do Cabo (Rio de Janeiro, Brazil) were collected and one was fixed in 70% ethanol, one in 4% formaldehyde, one dried and one was frozen.

Extraction of the sulfated polysaccharides

Each specimen was cut into small pieces (1 mm^3), washed with 70% ethanol, immersed tree times in acetone and dried at a 60°C oven. Sulfated polysaccharides were extracted from the dried tissues (100 mg from *C. australiensis* and *C. nucula*, and 30 mg from all the other specimens) by extensive papain digestion, and the extracts were partially purified by cethylpyridinium and ethanol precipitations using the same methodology described for other invertebrate tissues (Vieira *et al.* 1991). Approximately 3 mg (dry weight) of crude extract was obtained from *C. australiensis* and *C. nucula* and 1 mg from the other species.

Purification of the sulfated polysaccharides

The crude extracts of sulfated polysaccharides (3 mg of each specimen) were applied to a DEAE cellulose column, equilibrated with 5 mM sodium acetate (pH 5.0) with 10 mM EDTA (ethylenediaminetetraacetic acid). The polysaccharides were eluted from the column using a linear gradient of 0-3 M NaCl, at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and checked by metachromatic assay using 1,9-dimethylmethylene blue (Farndale *et al.* 1986), and by measuring conductivity. The fractions containing sulfated polysaccharides were pooled, dialyzed against distilled water and lyophilized.

Agarose gel electrophoresis

The crude extracts and purified sulfated polysaccharides were analyzed by agarose gel electrophoresis. The sulfated polysaccharides (15 µg) were applied to a 0.5% agarose gel and run for 1 h at 110 V in a 0.05 M 1,3-diaminopropane-acetate buffer (pH 9.0). The sulfated polysaccharides in the gel were fixed with 0.1% *N*-cetyl-*N*,*N*,*N*trimethylammonium bromide solution. After 12 h, the gel was dried and stained with 0.1% toluidine blue in 0.1:5:5 acetic acid:ethanol:water.

Polyacrylamide gel electrophoresis

The molecular masses of the sulfated polysaccharides were estimated by polyacrylamide gel electrophoresis. Sulfated polysaccharides (15 µg) were applied to a 6% 1 mm thick polyacrylamide gel slab in 0.02 M sodium barbital (pH 8.6). After electrophoresis (100 V for 30 min), the gel was stained with 0.1% toluidine blue in 1% acetic acid and then washed for about 4 h in 1% acetic acid. The molecular mass markers were the low-molecular-mass dextran sulfate (8 kDa), chondroitin 4-sulfate from shark cartilage (40 kDa) and high-molecular-mass dextran sulfate (500 kDa).

Chemical analysis

Total hexose and uronic acid were estimated by the phenol–H_2SO_4 reaction (Dubois *et al.* 1956) and carbazole reaction (Dische 1947), respectively. After acid hydrolysis of the polysaccharides (6.0 M trifluoroacetic acid for 5 h at 100°C), sulfate was measured by the $BaCl_2$–gelatin method (Saito *et al.* 1958). The presence of different neutral sugars was estimated by paper chromatography in 3:2:1 *n*-butanol–pyridine–water for 48 h (Kircher 1954).

Results

Fractionation of the sulfated polysaccharides from *C. australiensis* resulted in a single and sharp peak, eluted with 0.5 M NaCl (Fig. 1A). *C. nucula* showed a single sulfated polysaccharide fraction too, but eluted with a higher NaCl concentration of 1M (Fig. 1B). These results indicate the presence of a single and homogeneous population of sulfated polysaccharides in the two species.

The presence of a single population of sulfated polysaccharides and the purity of the fractions were confirmed by agarose gel electrophoresis. The sulfated polysaccharides from two specimens of either *C. australiensis* or *C. nucula* showed narrow bands with similar electrophoretical motility (Fig. 2). However, the electrophoretical motility of the sulfated polysaccharides from *C. australiensis* and *C. nucula* differs significantly (Fig. 2), which indicates that *C. australiensis* and *C. nucula* have distinct sulfated polysaccharides. Polyacrylamide gel electrophoresis showed that sulfated polysaccharides from *C. australiensis* and *C. nucula* have high molecular weights (approximately 500 kDa; Fig. 3).

The differences between the sulfated polysaccharides from *C. australiensis* and *C. nucula* were evaluated by their sugar compositions, as well as, hexuronic acid and sulfate contents. The sulfated polysaccharides from *C. australiensis* and *C. nucula* showed the same sugar composition and similar hexuronic acid content (Table 2). However, the sulfate content of the sulfated polysaccharides from *C. nucula* was approximately 50% higher than that from *C. australiensis* (Table 2). Therefore, the major difference between the sulfated polysaccharides from *C. australiensis* and *C. nucula* is their sulfate content.

The crude extract of sulfated polysaccharides from two specimens of *C. nucula* from France, two specimens of *Chondrilla* sp. B from Brazilian coast and two specimens of *Chondrilla* sp. E and two of *Chondrilla* sp. F from Bahamas were applied to an agarose gel electrophoresis (Fig. 4).

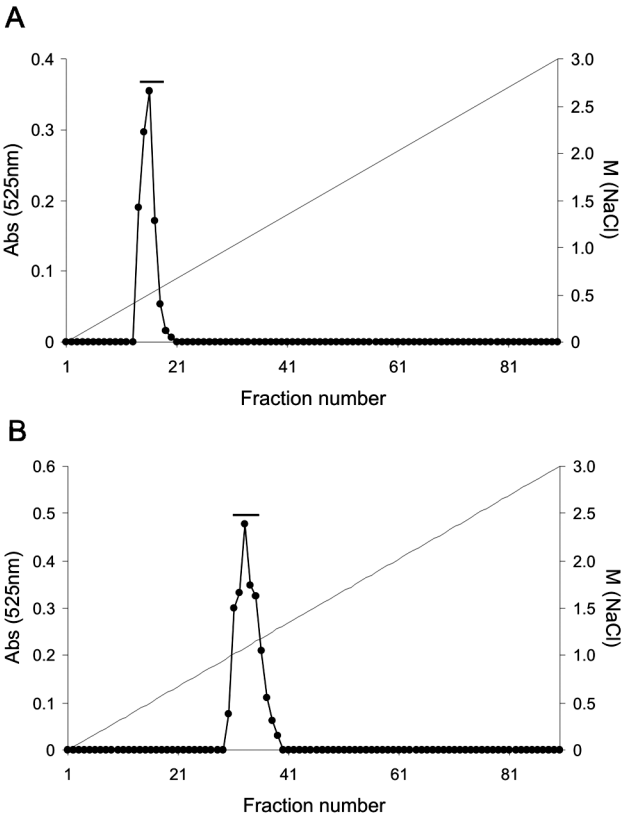

Fig. 1: Purification of the sulfated polysaccharides. The crude extracts of sulfated polysaccharides from *C. australiensis* (**A**) and *C. nucula* (**B**) (~3mg each) were purified by ion exchange chromatography (DEAEcellulose-FPLC). The samples were eluted by a linear gradient of 0–3 M NaCl. The fractions were checked by its metachromatic property (•) and NaCl concentration (—). The fractions indicated by the horizontal bar corresponding to the purified sponge sulfated polysaccharides.

The sulfated polysaccharides from these cryptic species of *Chondrilla* showed distinct electrophoretical motilities. These differences in the electrophoretical motility show that the agarose gel electrophoresis of crude extract of sulfated polysaccharides has a good resolution to distinguish cryptic species of *Chondrilla*.

In order to evaluate the effectiveness of the method for samples of sponges fixed in different media, we extracted sulfated polysaccharides from specimens of *Chondrilla* sp. B fixed either in 70% ethanol, 4% formaldehyde, frozen at -20°C or dried at 60°C. The electrophoretic motility of the sulfated polysaccharide was the same, independent of the method used to fix the sponge (Fig. 5).

Discussion

In the present study we show a new, simple and efficient method to distinct cryptic species within *Chondrilla*. We also demonstrate that this methodology can be performed with specimens fixed in formaldehyde, a preservative that

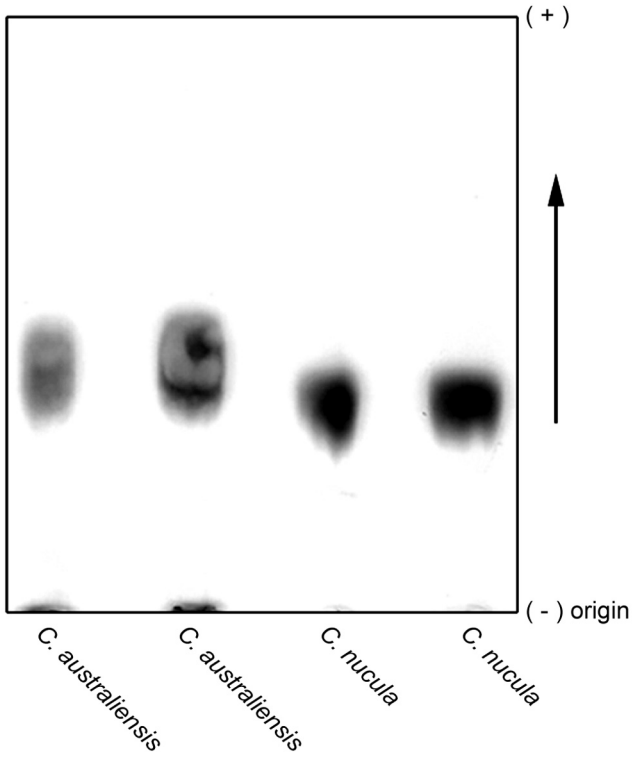

Fig. 2: Agarose gel electrophoresis of the purified sulfated polysaccharides from *C. australiensis* and *C. nucula* (~15 µg of each).

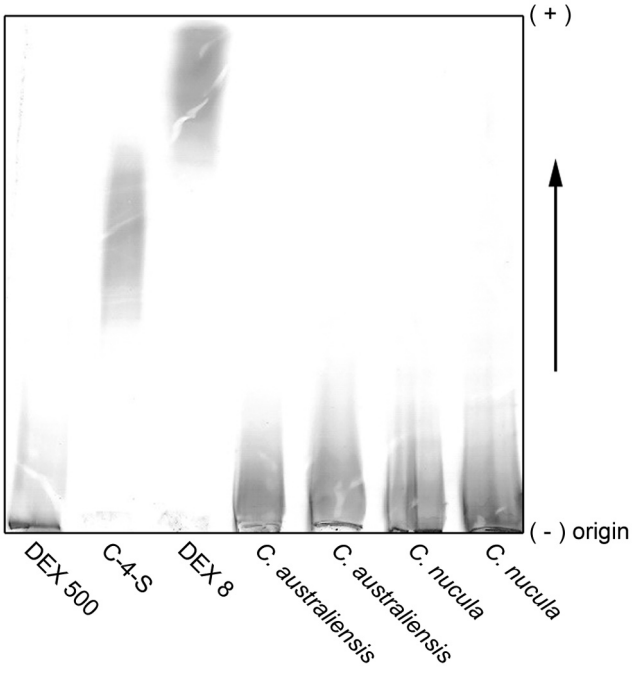

Fig. 3: Polyacrylamide gel electrophoresis of the purified sulfated polysaccharides from *C. australiensis* and *C. nucula* (~15 µg of each). The molecular mass markers were high-molecular-mass dextran sulfate (Dex500, 500 kDa), chondroitin 4-sulfate from whale cartilage (C-4-S, 40 kDa), and low-molecular-mass dextran sulfate (Dex8, 8 kDa).

Table 2: Chemical composition of the sulfated polysaccharides from *C. nucula* and *C. australiensis*.

Species	Sugar composition[a]	Total hexose[b,d]	Total sulfate[b,d]	Hexuronic acid[c,d]	Sulfate/ total hexose[c]
C. australiensis	Ara, Gal, Fuc and HexUA	2.19	2.16	0.12	0.99
C. nucula		2.44	3.73	0.10	1.53

[a] The sugar composition was determined by paper chromatography of hydrolyzed sulfated polysaccharides. [b] nMoles/ml. [c] Molar ratio. [d] Total hexose, total sulfate and hexuronic acid were measured by phenol-sulfuric acid, $BaCl_2$-gelatin and carbazole methods, respectvely.

impounds the use of allozymes or even DNA sequencing analyses.

The sulfated polysaccharides from *C. australiensis* and *C. nucula* showed high molecular weight (~500 kDa), the same sugar composition (hexuronic acid, Ara, Fuc and Gal) and similar hexuronic acid content (12% and 10%, respectively). The only chemical difference between the sulfated polysaccharides from these species was the sulfate: total sugar molar ratio (1:1 and 1.5:1, respectively). Zierer and Mourão (2000) reported the chemical characterization of *C. nucula* from Arraial do Cabo, Brazil. This species is actually a cryptic species of *C. nucula* temporarily named *Chondrilla* sp. B (Klautau *et al.* 1999). The sulfated polysaccharide from *Chondrilla* sp. B contains hexuronic acid, Ara, Fuc and Gal, the hexuronic acid accounts for 25% of the total sugar and the sulfate:total sugar molar ratio is 2.5:1 (Zierer and Mourão 2000). Therefore, the differences detected among the sulfated polysaccharides from *C. australiensis*, *C. nucula* and *Chondrilla* sp. B were mostly related to their sulfate and hexuronic acid content. This confirms the species specific composition of the sulfated polysaccharides from sponges, even among species of the same genus.

Differences among sulfated polysaccharides from congeneric species have also been observed in the α-L-fucans isolated from the jelly coat of eggs of four sea urchin species within the genus *Strongylocentrotus* (Alves *et al.* 1998). The differences among these fucans are only in the sulfate pattern and the position of the glycosidic bonds (Alves *et al.* 1998, Villela-Silva *et al.* 1999, 2001). These structural differences of sulfated polysaccharides are sufficient to avoid interspecific fertilization among these congeneric species (Biermann *et al.* 2004). The sulfated polysaccharides from the three species of

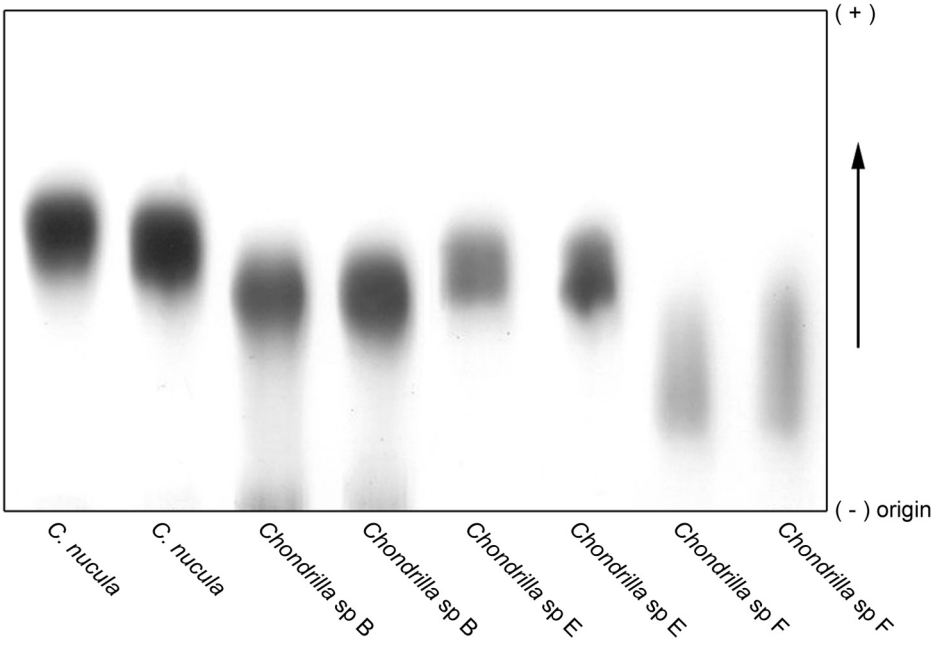

Fig. 4: Agarose gel electrophoresis of the crude extracts of sulfated polysaccharides from *Chondrilla nucula*, *Chondrilla* sp. B, and the two sympatric species *Chondrilla* sp. E and *Chondrilla* sp. F (15 µg of each).

Chondrilla also showed differences in their sulfation pattern. However, we still need a structural characterization of these sulfated polysaccharides, such as the position of glycosidic bonds and sulfation sites to determine all their differences.

The electrophoretic motility of sulfated polysaccharides in agarose gel is mostly determined by their interaction with 1,3-diaminopropane, which depends on the structure and sulfation pattern of the sulfated polysaccharide (Dietrich and Dietrich 1972, 1976). This methodology had enough resolution to separate sulfated polysaccharides with small structural differences. For instance, it can separate the glycosaminoglicans dermatan sulfate and condroitin-4-sulfate, which differs exclusively on the type of hexuronic acid in the chains (glycuronic acid in chondroitin-4-sulfate and iduronic acid in dermatan sulfate) (Dietrich and Dietrich 1972, 1976). Therefore, agarose gel electrophoresis in 1,3-diaminopropane buffer can be used to distinguish sulfated polysaccharides with small structural differences.

Chondrilla nucula was once considered as cosmopolitan. However, a study using allozymes electrophoresis showed that *C. nucula* was in fact a complex of cryptic species (Klautau *et al.* 1999). In the present study, four cryptic species of *Chondrilla* detected through allozymes by the presence of at least one diagnostic locus (Zilberberg 2006, Zilberberg *et al.* 2006) were analyzed through their sulfated polysaccharides. The four species were separated by their sulfated polysaccharides, including the two sympatric and cryptic Bahamian species (named sp. E and sp. F; Zilberberg *et al*. 2006). This result demonstrates the good resolution of the technique to separate cryptic species of *Chondrilla*. Additionally sulfated polysaccharides analyses by agarose gel electrophoresis showed some advantages in relation to allozymes or DNA analyses. Allozyme electrophoresis techniques require fresh or frozen samples (Wörheide *et al*. 2004). Therefore, DNA sequencing analyses were advantageous over allozymes by the ability to work with

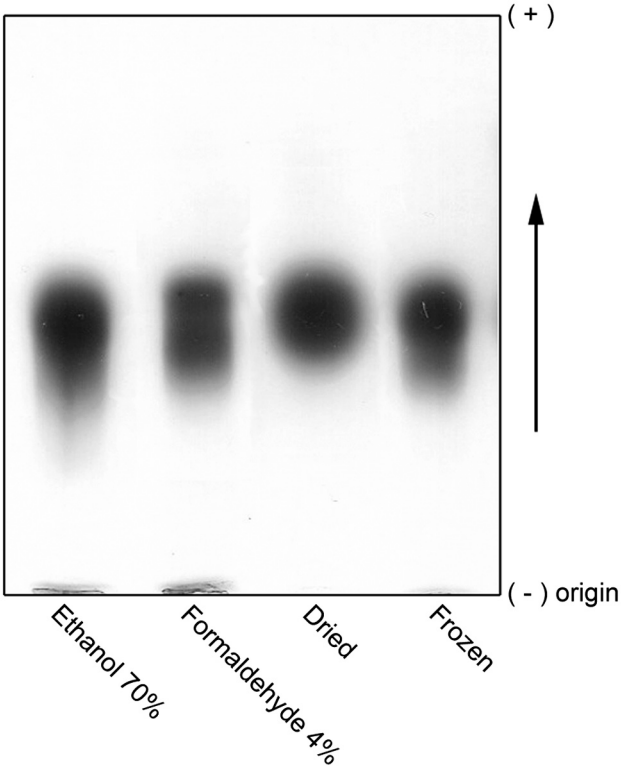

Fig. 5: Agarose gel electrophoresis of the crude extracts of sulfated polysaccharides from specimens of *Chondrilla* sp. B fixed in ethanol 70%, formaldehyde 4%, dried and frozen (~15 µg of each).

dried or ethanol preserved specimens. However, DNA sequencing analyses are unfeasible with tissues preserved in formaldehyde, and most of the earlier preserved museum specimens used this fixative. Thus, sulfated polysaccharides

analyses have some advantages over allozymes and DNA, since it is a quick (~ 5 days) and very low cost technique (~ US$ 2.00 / sample), it requires a very small sample (30mg of sponge tissue), and most importantly, it can be performed in formaldehyde preserved specimens.

We can conclude that the analysis of the sulfated polysaccharides by agarose gel electrophoresis is a promissory biochemical technique to distinguish cryptic species within Porifera. However, further analyses with a larger sample size, a higher number of cryptic species of *Chondrilla* and other sponge taxa must be performed to establish the general effectiveness and robustness of this technique.

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