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## High diversity of frenulates (Polychaeta: Siboglinidae) in the Gulf of Cadiz mud volcanoes: A DNA taxonomy analysis

A. Hilário<sup>a,\*</sup>, S.B. Johnson<sup>b</sup>, M.R. Cunha<sup>a</sup>, R.C. Vrijenhoek<sup>b</sup>

<sup>a</sup> CESAM, Departamento de Biologia, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

<sup>b</sup> Monterey Bay Aquarium Research Institute, Moss Landing, California 95039-9644, USA

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## ABSTRACT

Frenulates are the most poorly known members of the family Siboglinidae (Polychaeta: Canalipalpata). These thread-like worms occur in reducing marine sediments worldwide, but they are often overlooked in benthic samples or too poorly preserved for adequate taxonomic evaluations. We report on a remarkable diversity of frenulates that were recently sampled from 13 mud volcanoes (350–3902 m deep) in the Gulf of Cadiz, off southern Iberia. Sampled with benthic coring devices, the bodies of these long tubiculous worms were often broken or incomplete, making them difficult to identify morphologically. Consequently, we employed DNA taxonomic methods to assess their diversity. Mitochondrial cytochrome-*c*-oxidase subunit 1 (*COI*) sequences distinguished 15 evolutionary lineages inhabiting the Gulf of Cadiz. Only four of the lineages could be assigned to currently recognized Atlantic species; the remaining 11 may be new to science. This remarkable diversity of frenulates in a small geographical region is unprecedented and is hypothesized to result from environmental heterogeneity associated with the bathymetric and geochemical settings of these mud volcanoes.

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### 1. Introduction

The annelid family Siboglinidae (Polychaeta: Canalipalpata) contains approximately 160 nominal species that inhabit deep-sea reducing environments. Named after the *Siboga* Expedition during which they were first collected (Caullery, 1914), these tubeworms are among the dominant constituents of the invertebrate communities at hydrothermal vents, cold-water sulphide/hydrocarbon seeps, and whale-falls worldwide (Braby et al., 2007; Southward et al., 2005; Tunnicliffe et al., 2003). Adult siboglinids lack a digestive system and depend entirely on endosymbiotic bacteria for their nutrition (reviewed in Thornhill et al., 2008). Most species have long and thin bodies that span oxic–anoxic boundaries in the marine benthos, absorbing oxygen with an anterior gill-like structure and reduced compounds (e.g., hydrogen sulfide, methane or organic compounds) through their posterior body (Goffredi et al., 2005; Southward et al., 2005). Vestimentiferans, which dominate hydrothermal vents and cold seep environments in the eastern Pacific and Gulf of Mexico, are the most extensively studied siboglinids (Tunnicliffe et al., 2003). Bone-eating worms of the genus *Osedax*, which were first reported in 2004, have been the subject of intense interest (Fujikura et al., 2006; Glover et al., 2005b; Rouse et al., 2004, 2008; Southward

et al., 2005). Vestimentiferans are relatively easy to collect with the aid of submersibles because they are large and often occur in clumps. Though *Osedax* are small, they occur on bones that are also relatively easy to sample with submersibles. *Sclerolinum* and the frenulates, on the other hand, are mostly small thread-like worms that are buried deeply in soft sediments. Their *in situ* observation is very difficult, so they are usually sampled as by-catch in benthic cores. Consequently, they are often poorly preserved or simply overlooked (Halanych, 2005).

The systematic position and nomenclature of Siboglinidae Caullery (1944) was in a state of flux for many years (reviewed in Pleijel et al., 2009). Pogonophorans and vestimentiferans have at various times been elevated to the rank of phylum, but current morphological, embryological and molecular evidence places these worms firmly within the Polychaeta (Black et al., 1997; Halanych, 2005; Halanych et al., 2001; Kojima et al., 1993; McHugh, 1997; Rouse, 2001; Rouse and Fauchald, 1997; Southward, 1999; Young et al., 1996). As a result of these studies, most researchers now recognize the family Siboglinidae as encompassing three discrete clades: the Frenulata; the Monilifera (=Vestimentifera+*Sclerolinum* as defined by Rouse (2001)); and *Osedax* (Rouse et al., 2004). Nonetheless, some researchers do not recognize the new classification and continue to use the names Pogonophora and Vestimentifera to represent annelid classes (e.g., Bartolomaeus et al., 2005; Southward et al., 2005).

The present study was stimulated by the discovery of biologically diverse chemosynthetic communities in the Gulf of

\* Corresponding author. Tel.: +351 234370969; fax: +351 234372586.  
E-mail address: [ahilario@ua.pt](mailto:ahilario@ua.pt) (A. Hilário).

Cadiz, off the southern Iberian Peninsula (Cunha et al., 2001; Rodrigues and Cunha, 2005). Ongoing explorations conducted by several research programmes (Akhmentzhanov et al., 2007; Weaver et al., 2004) have provided excellent opportunities to sample the fauna and extend our knowledge of the frenulates found at several mud volcanoes in this region. Based on their unique morphological characteristics, a new frenulate genus, *Bobmarleya*, and two new species, *Bobmarleya gadensis* and *Spirobrachia tripeira*, have already been described from the Gulf of Cadiz (Hilário and Cunha, 2008). Morphological studies alone are not sufficient, however, to provide a precise accounting of frenulate species diversity in this region. Several putatively new species can only be distinguished by subtle morphological characters that are easily damaged during collection and dissection. Furthermore, some diagnostic traits tend to vary with the age of individuals (Southward, 1969). Consequently, we employ DNA taxonomic methods in the present study because they can be used to circumscribe and delineate distinct evolutionary lineages from incomplete or damaged individuals and from various life-history stages (Vogler and Monaghan, 2007). This approach has proved immensely useful for identifying morphologically cryptic species in several poorly known deep-sea taxa (Vrijenhoek, 2009). We employ invertebrate primers that amplify DNA sequences from mitochondrial cytochrome-c-oxidase subunit 1 (*COI*) (Folmer et al., 1994; Nelson and Fisher, 2000). This gene has already been used successfully in a number of studies to delineate vestimentiferan (Chevaldonné et al., 2002; Feldman et al., 1998; Kojima et al., 2002) and *Osedax* species (Braby et al., 2007; Fujikura et al., 2006; Jones et al., 2008). Here we use *COI* sequences to distinguish among 15 discrete evolutionary lineages of frenulates from the Gulf of Cadiz mud volcanoes.

## 2. Materials and methods

### 2.1. Sample collection and preservation

For comparative purposes we included a specimen of *Spirobrachia* cf. *grandis* that was provided and identified by Eve Southward. All other specimens were collected from 13 mud volcanoes in the Gulf of Cadiz during several recent cruises (Table 1, Fig. 1) and preserved in 96% ethanol. The animals were removed from their tubes and a portion of the trunk was used for *COI* sequencing. Preliminary identifications of specimens, based on tube and soft tissue morphology, were conducted with the help of Eve Southward (Marine Biological Association of the United Kingdom). Complete vouchers for all of the 15 lineages found in this study were preserved in 4% seawater formalin and deposited in the Biological Research Collection of Marine Invertebrates of the University of Aveiro (DBUA) for future comparative morphology and descriptive studies (DBUA00960-DBUA00980).

### 2.2. Cytochrome oxidase sequencing

Genomic DNA was isolated using the Qiagen DNeasy DNA extraction kit following the manufacturer's protocol (Qiagen Inc., Valencia, CA). Segments of approximately 1200 and 650 base-pairs (bp) of mitochondrial cytochrome-c-oxidase subunit I (*COI*) were amplified with primers based on regions conserved in invertebrates (Folmer et al., 1994; Nelson and Fisher, 2000). PCR was conducted in 25  $\mu$ l reactions that included 30–100 ng of template DNA, 2.5  $\mu$ l of  $\times 1$  of PCR buffer (supplied by manufacturer), 2.5  $\mu$ l of 2.5  $\mu$ M MgCl<sub>2</sub>, 1  $\mu$ l of each primer (10 mM final conc.), 2.5 units Amplitaq Gold DNA<sup>®</sup> polymerase (Applied Biosystems, Foster City, CA), 2.5  $\mu$ l of 2 mM stock

solution of dNTPs, and sterile water to final volume. Amplifications for all loci, which occurred with a Cetus 9600 DNA Thermal Cycler (Perkin-Elmer Corp., CT), used an initial denaturation of 95 °C/10 min, followed by 35 cycles of 94 °C/1 min, 55 °C/1 min, and 72 °C/1 min, and a final extension at 72 °C/7 min. PCR products were diluted in 40  $\mu$ l sterile water and cleaned with Multiscreen HTS PCR 96 Filter plates on a vacuum manifold (Millipore Corp., Billerica, MA). PCR products were sequenced bidirectionally with the same primers used in PCR on an ABI 3100 capillary sequencer using BigDye terminator v3.1 chemistry (Applied Biosystems Inc., Foster, California). DNA sequences were proofread using Sequencher v 4.7 (Gene Codes Corp. Inc., Ann Arbor, Michigan) and aligned and edited by eye with MacClade v4.08 (Maddison and Maddison, 2005).

### 2.3. Phylogenetic analyses

Published sequences (Table 2) from *Osedax rubiplumus*, *Sclerolinum brattstromi* and *Lamellibrachia columna* were used to represent the outgroup taxa in phylogenetic analyses. The analyses were conducted with the program Mr. BAYES v. 3.1.2 (Ronquist and Huelsenbeck, 2003). An appropriate substitution model for *COI* was determined using the MrModelTest2 procedure (Nylander, 2004) within the program PAUP\* v. 4.02 (Swofford, 2002). The site-specific general time reversible substitution model (GTR+SS) provided the best fit for the data obtained in this study. Bayesian analyses utilized six chains, conducted for  $5 \times 10^7$  generations with a print and sampling frequency of 1000, and a burn-in of 2500. Each analysis was repeated five times and data were visualized using TRACER v. 1.3 (Rambaut and Drummond, 2003) and AWTY (Wilgenbusch et al., 2004) to determine the appropriate burn-in and ensure that the data had reached convergence. Trees were visualized with FIGTREE v. 1.2.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). Saturation plots of nucleotide substitutions were created using the program DAMBE v. 5.0.25 (Xia and Xie, 2001). The plots (not shown) revealed significant saturation for transitions but not for transversions.

### 2.4. Gauging MOTUs

The 5'-end of *COI* has become the main barcoding gene because it is sufficiently variable to differentiate among the named species of many animal phyla (Hebert et al., 2003). Here, we adopt a  $D_a$  value of 4% as a practical guide for the recognition of molecular operational taxonomic units (MOTUs, sensu Blaxter et al., 2005), and consider them as worthy of further investigation as putative species. We used PAUP\* v. 4.02 (Swofford, 2002) to estimate pairwise distances between *COI* sequences ( $p$ -distances= $n_d/L$ , where  $n_d$  is the absolute number of differences and  $L$  is sequence length).

## 3. Results

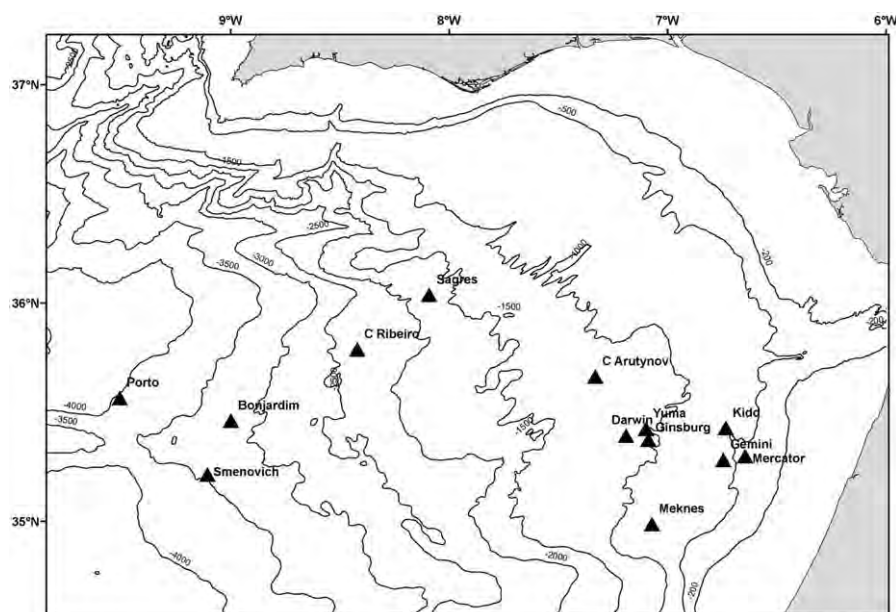
Altogether, 53 new *COI* sequences, ranging from 448 to 850 base-pairs (bp) in length, allowed us to distinguish among 15 frenulate MOTUs from the Gulf of Cadiz. *Spirobrachia* cf. *grandis* from the Aleutian Fan in the North Pacific was included in this group for comparative purposes. Uncorrected nucleotide divergence ( $p$ -distance) was estimated between all pairs of sequences and assembled into estimates of mean divergence within ( $D_w$ ) and among ( $D_a$ ) the MOTUs (Table 3). Where multiple sequences were obtained, the  $D_w$  values within MOTUs were less than or equal to 0.6%, a value that was much less than the smallest  $D_a$  values (4.7%) among the frenulate MOTUs.

**Table 1**

Collection data for samples used for the molecular analysis.

Mud volcano (abbr.)	Expedition	Station	Sample method	Latitude N	Longitude W	Depth (m)	Specimen ID <sup>a</sup>	N
Mercator (Mer)	MSM01-03	267	M	35°17.875'	06°38.789'	350	Mer(350)	4
	MSM01-03	241	B	35°17.918'	06°38.717'	353	Mer(353)	1
	TTR15	AT575	B	35°17.903'	06°38.715'	355	Mer(355)	1
Gemini (Gem)	Microsys. 2006	M2006-10	B	35°16.830'	06°45.540'	432	Gem(432)	1
		TTR14	AT528	G	35°25.304'	06°43.972'	489	Kid(489)
Kidd (Kid)	TTR14	AT528	G	35°25.304'	06°43.972'	489	Kid(489)	2
Meknes (Mek)	MSM01-03	319	B	34°59.100'	07°04.439'	695	Mek(695)	4
Yuma (Yum)	TTR16	AT605	G	35°25.046'	07°05.450'	975	Yum(975)	2
Ginsburg (Gin)	TTR16	AT607	G	35°22.677'	07°04.979'	983	Gin(983)	4
Darwin (Dar)	TTR16	AT608	G	35°23.531'	07°11.475'	1115	Dar(1115)	4
	JC10	036PUC09	P	35°23.541'	07°11.508'	1112	Dar(1112)	1
Cap. Arutyunov (Cap)	MSM01-03	190	M	35°39.668'	07°19.970'	1320	Cap(1320)	1
	MSM01-03	194	G	35°39.282'	07°20.012'	1401	Cap(1401)	2
Sagres (Sag)	TTR17	AT667	G	36°02.199'	08°05.545'	1562	Sag(1562)	2
Carlos Ribeiro (Car)	TTR16	AT615	G	35°47.238'	08°25.272'	2200	Car(2200)	3
	JC10	051PUC02	P	35°47.244'	08°25.282'	2197	Car(2197)	1
Bonjardim (Bon)	TTR15	AT597	G	35°27.563'	09°00.030'	3061	Bon(3061)	2
	MSM01-03	133	B	35°27.821'	09°00.128'	3049	Bon(3049)	3
	TTR17	AT676	G	35°27.527'	08°59.828'	3062	Bon(3062)	1
Smenovich (Sme)	TTR17	AT679	G	35°13.433'	09°05.186'	3265	Sme(3265)	1
Porto (Por)	TTR16	AT622	G	35°33.773'	08°30.416'	3902	Por(3902)	12
	TTR17	AT687	G	35°33.774'	09°30.429'	3887	Por(3887)	1

B—Box core; G—TV-grab; M—Mega core; P—Push core. Mud volcanoes ordered by depth.

<sup>a</sup> Specimen identifier with sample depth in parenthesis.**Fig. 1.** Map showing the mud volcanoes from which samples were collected.

Bayesian phylogenetic analysis of the present sequences resolved five well-supported basal clades within the frenulates (Groups I–V, Fig. 2). Hierarchical relationships among the groups were not adequately resolved with these *COI* sequences. The Bayesian posterior probabilities were  $\leq 0.62$  for basal nodes, so we collapsed them into an unresolved polytomy. Given the observed degree of saturation at 3rd-position transitions, these sequences do not provide a sound basis for inferring hierarchical relationships among the five groups. All but one of the five groups (III) contained multiple evolutionary lineages with genetic

distances ( $D$ ) greater than 4%; so, we treat them subsequently as discrete MOTUs.

Group I is the most diverse of the five clades, containing six MOTUs (labelled a–f, Fig. 2). Sequence divergence among these MOTUs ranged from a low  $D_a$  value of 4.5% (*I.e* vs. *I.f*) to a high  $D_a$  of 20.0% (*I.a* vs. *I.f*). The  $D_w$  values did not exceed 1.4% though sample sizes were very small ( $n=2$ ) in three MOTUs. The group I lineages covered the full range of depths that were sampled. Two MOTUs had wide depth ranges, *I.b* (350–3061 m) and *I.f* (695–3902 m). MOTU *I.a* may have the narrowest range

**Table 2**  
Molecular operational taxonomic units (MOTUs) ascertained from analysis of mitochondrial *COI* sequences.

Group	MOTU	Acc. nos.	Source <sup>a</sup>
I	<i>I.a</i>	<b>FJ480347–FJ480355</b>	Yum, Gin, Dar
	<i>I.b</i>	<b>FJ480378–FJ480387</b>	Mer, Gem, Kid, Bon
	<i>I.c</i>	<b>FJ480394–FJ480395</b>	Bon
	<i>I.d</i>	<b>FJ480396–FJ480397</b>	Mer, Cap
	<i>I.e</i>	<b>FJ480358–FJ480359</b>	Dar
	<i>I.f</i>	<b>FJ480364–FJ480369</b>	Mek, Por
II	<i>Siboglinum cf. poseidoni</i>	<b>FJ480398–FJ480399</b>	Cap
	<i>II.a</i>	<b>FJ480360–FJ480363</b>	Car, Por
III	<i>Bobmarleya gadensis</i>	<b>FJ380356–FJ380357</b>	Car
IV	<i>Spirobrachia tripeira</i>	<b>FJ480370–FJ480375</b>	Por
	<i>Spirobrachia cf. grandis</i>	<b>FJ483547</b>	Aleutian Fan <sup>b</sup>
	<i>Lamellisabella denticulata</i>	<b>FJ480376–FJ480377</b>	Por
	<i>IV.a</i>	<b>FJ480388</b>	Bon
V	<i>Galathealinum brachiosum</i>	U744066	Black et al. (1997) <sup>c</sup>
	<i>V.a</i>	<b>FJ480389–FJ480390</b>	Sme, Car
	<i>V.b</i>	<b>FJ480391–FJ480395</b>	Sag
	<i>V.c</i>	<b>FJ480393</b>	Bon
Outgroups	<i>Osedax rubiplumus</i>	DQ996618	Braby et al. (2007)
	<i>Sclerolinum brattstromi</i>	<b>FJ347644</b>	off Bergen, Norway
	<i>Lamellibrachia columna</i>	DQ996645	Braby et al. (2007)

All GenBank accession numbers in boldface are original to this study.

<sup>a</sup> Unless otherwise noted, the 3-letter code represents the mud volcano from Gulf of Cadiz, see Table 1.

<sup>b</sup> From the Aleutian Trench (57°27.3949N, 148°00.0139W; 4890 m depth).

<sup>c</sup> From Oregon Subduction Zone (45°01'N; 125°19'W; 2028 m depth).

(975–1115 m). Less can be said about lineages *I.c* (3049 m) and *I.d* (1115 m), as only two individuals of each lineage were sampled from single mud volcanoes. Two individuals also represented lineage *I.d*, but they were sampled from different depths of 353 and 1320 m. All of group *I* lineages have ringed tubes, with simple or anastomosing rings. The presence of a single anterior tentacle and well-developed metameris papillae on the preannular region of the trunk reveals a possible affiliation with the genus *Siboglinum* as defined by Ivanov (1963), but as shown below, group *I* appears to be paraphyletic with respect to group *II*, which contains *Siboglinum cf. poseidoni*.

Group *II* contains two MOTUs that differ by 22.0% on average (Table 3). Two specimens identified conservatively as *S. cf. poseidoni* were sampled from Captain Arutyunov mud volcano at 1320–1401 m. *S. poseidoni* is known from shelf depths in the northeast Atlantic, but specimens from the type locality (Skagerrak Strait, off Sweden) were not available for molecular comparisons. Four specimens of lineage *II.a* were sampled at 2200–3902 m. The structure of their tube is simple, without segmentation or rings, resembling that of the genus *Cyclobrachia* (Ivanov, 1960). Although most of the specimens were too fragmentary, it was possible to distinguish more than 10 tentacles with small pinnules.

Group *III* contains specimens of *B. gadensis* (Hilário and Cunha, 2008) sampled from the type locality, the Carlos Ribeiro mud volcano at ~2200 m.  $D_w$  from the two specimens was 0.4%.

Group *IV* contains four MOTUs that were previously assigned to the genera *Spirobrachia* and *Lamellisabella* (Hilário and Cunha, 2008). Six *S. tripeira* (Hilário and Cunha, 2008) individuals were sampled from the Porto mud volcano (3902 m). They are relatively closely related to *S. cf. grandis* from the Aleutian Fan,  $D_a=7.2\%$ . Two individuals from the Porto mud volcano were identified as *Lamellisabella denticulata* (Hilário and Cunha, 2008). A single specimen representing the divergent MOTU *IV.a* was sampled from Bonjardim mud volcano. Its high degree of divergence from *L. denticulata* ( $D_a=15.6\%$ ) and morphological

characteristics of the tube, tentacular crown, anterior part of the trunk and girdles region suggest that it may represent a distinct *Lamellisabella* species.

Group *V* contains few samples that represent three divergent MOTUs ( $D_w$  range 10.5–13.2%) that were sampled from deeper waters (1562–3265 m). Their closest known relative, based on these *COI* sequence divergence, is *Galathealinum brachiosum*, a northeastern Pacific species. Preliminary morphological assessments suggested that *V.a* and *V.b* might be members of the genus *Polybrachia*. Their tube is segmented with funnel-like frills, the tentacular crown is formed by a large number of free tentacles, the mesosoma has several secondary annuli in front of the bridle and the forepart of the trunk bears cuticular plaques. A single specimen possessing only a single tentacle (*V.c* from Bon3049) could not be associated with any named genus or species.

## 4. Discussion

### 4.1. Molecular versus morphological to frenulate diversity

Biodiversity studies in the Gulf of Cadiz led to the discovery of an unprecedented diversity of frenulates, which could not be completely accessed by morphological studies. Sound taxonomy is an essential component of biodiversity studies; nevertheless, a serious shortage of taxonomic expertise exists throughout the scientific community (Buyck, 1999; Hopkins and Freckleton, 2002; Kim and Byrne, 2006; Wheeler, 2008). This shortage is evident with respect to frenulates because only three authorities have produced more than one-third of the publications on this group between 1949 and 2005 (including species descriptions, biogeography, phylogeny, functional morphology, ecology, symbiosis, etc.). Detailed morphological analyses and considerable expertise are needed to identify these worms, but adequate samples of their larval, juvenile, and adult stages are often incomplete because benthic cores contain only body segments.

**Table 3**  
Sequence divergence within ( $D_w$ , on diagonal) and between ( $D_a$ ) molecular operation taxonomic units (MOTUs).

MOTU (named species)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1 <i>I.a</i>	0.014																			
2 <i>I.b</i>	0.112	0.025																		
3 <i>I.c</i>	0.085	0.109	$n/e^a$																	
4 <i>I.d</i>	0.193	0.109	0.079	0.008																
5 <i>I.e</i>	0.193	0.184	0.165	0.172	0.001															
6 <i>I.f</i>	0.200	0.170	0.161	0.148	0.045	0.003														
7 <i>II</i> ( <i>Siboglinum</i> cf. <i>poseidoni</i> )	0.203	0.269	0.225	0.232	0.259	0.218	0.004													
8 <i>II.a</i>	0.218	0.269	0.225	0.232	0.259	0.218	0.220	$n/e^a$												
9 <i>III</i> ( <i>Bobmarleya gadensis</i> )	0.230	0.216	0.216	0.192	0.207	0.206	0.202	0.202	0.004											
10 <i>IV</i> ( <i>Spirobrachia tripeira</i> )	0.190	0.210	0.205	0.207	0.209	0.199	0.205	0.187	0.188	0.002										
11 <i>IV</i> ( <i>Spirobrachia</i> cf. <i>grandis</i> )	0.212	0.207	0.201	0.195	0.199	0.198	0.200	0.190	0.174	0.072	$n/e^a$									
12 <i>IV</i> ( <i>Lamellisabella denticulata</i> )	0.204	0.221	0.216	0.214	0.224	0.206	0.168	0.205	0.183	0.156	0.147	0.006								
13 <i>IV.a</i>	0.214	0.220	0.191	0.209	0.225	0.212	0.206	0.227	0.201	0.194	0.175	0.156	$n/e^a$							
14 <i>V</i> ( <i>Galatheidinium brachiosum</i> )	0.181	0.180	0.162	0.176	0.231	0.237	0.169	0.213	0.161	0.184	0.169	0.162	0.170	$n/e^a$						
15 <i>V.a</i>	0.202	0.198	0.195	0.188	0.211	0.222	0.223	0.227	0.171	0.181	0.164	0.199	0.205	0.101	$n/e^a$					
16 <i>V.b</i>	0.200	0.198	0.188	0.189	0.218	0.219	0.191	0.234	0.166	0.214	0.211	0.186	0.195	0.093	0.132	0.006				
17 <i>V.c</i>	0.187	0.206	0.169	0.176	0.230	0.219	0.184	0.234	0.175	0.191	0.207	0.182	0.195	0.096	0.125	0.105	$n/e^a$			
18 <i>Osedax rubiplumum</i>	0.275	0.300	0.266	0.269	0.280	0.274	0.279	0.250	0.306	0.249	0.272	0.291	0.262	0.249	0.279	0.266	$n/e^a$			
19 <i>Sclerolinum brachiosum</i>	0.241	0.224	0.209	0.225	0.247	0.243	0.289	0.259	0.224	0.279	0.287	0.265	0.244	0.217	0.252	0.251	0.243	$n/e^a$		
20 <i>Lamellibrachia columna</i>	0.274	0.226	0.242	0.233	0.256	0.253	0.258	0.262	0.277	0.249	0.236	0.248	0.251	0.204	0.238	0.239	0.237	0.246	$n/e^a$	0.206

<sup>a</sup> Not estimated, sample size of one.  
<sup>b</sup> From Vrijenhoek et al. (in revision).

Molecular taxonomy provided a means to facilitate these identifications from incomplete samples of frenulates from the Gulf of Cadiz. The present *COI* sequences allowed us to distinguish 15 molecular operational taxonomic units. Four of these MOTUs could be assigned to nominal species. The smallest *COI* divergence between the 15 MOTUs was 4.5%. On average, the MOTUs differed from one-another by 16.9%. Among-species sequence divergence ( $D_a$ ) for *COI* typically exceeds 4% for a range of deep-sea annelids, whereas within-species divergence ( $D_w$ ) rarely exceeds 1% (Hurtado et al., 2002; Kojima et al., 2003; Rouse et al., 2004; Glover et al., 2005a; Wiklund et al., 2009). Thus, we are reasonably confident that the 11 unnamed MOTUs warrant further investigation as putative species. To determine whether the unnamed MOTUs are new to science, future studies should examine *COI* sequences from comparative materials. Though approximately 140 species of frenulates are currently recognized, only five have been included in previous molecular systematic studies (Halanych et al., 2001; Winnepenninckx et al., 1995). Hopefully, future deep-sea explorations will preserve samples that will allow molecular comparisons to be made.

The present phylogenetic analysis of *COI* sequences does not provide sound criteria for genus-level assignments of the the Gulf of Cadiz frenulates. The *COI* sequences were saturated with respect to 3rd-position transitions, so we could only portray a basal polytomy that included five discrete clades. Mitochondrial *COI* sequences alone have limited power to resolve higher-level relationships within families of deep-sea annelids and molluscs (reviewed in Vrijenhoek, 2009). Independent gene sequences from several nuclear gene loci will be needed to provide a robust phylogeny, and detailed morphological analyses of these and other frenulates are needed to revise genus-level assignments.

A combined molecular and morphological approach would be especially useful for the nominal genus *Siboglinum* (Caullery, 1914). Sixty-nine species, based on presence of a single tentacle, are currently assigned to this genus. Several multitentaculate species have a unitentaculate juvenile stage, so a number of nominal “*Siboglinum*” species may be misclassified (Southward, 1969). In the present study, the single unitentaculate specimen of *V.c* from Bonjardim mud volcano would have been assigned to *Siboglinum*, if the *COI* sequences had not placed it with the multitentaculate group *V*. It should also be noted that *S. cf. poseidoni* is separated in group *II*, apart from the other unitentaculate MOTUs clustered within group *I*. *S. poseidoni* is the only frenulate known to be hermaphroditic and to harbour methantrophic endosymbionts (Flügel and Langhof, 1983; Schmaljohann and Flügel, 1987), characteristics that may warrant reconsideration of its placement in this genus. Because the clade *II.a* is multitentaculate it is worth considering the possibility that the unitentaculate condition might be homoplastic.

#### 4.2. Mechanisms maintaining high frenulate diversity in the Gulf of Cadiz

Although the underlying mechanisms affecting the distribution of frenulates are not completely understood (Southward and Dando, 1988), we suspect that variation among mud volcanoes in geochemical settings and bathymetry may support their remarkable diversity in the Gulf of Cadiz. Because frenulates absorb reduced compounds through the posterior parts of their tubes, the oxidation–reduction (redox) profile of the sediment is expected to play a role in determining which species are able to inhabit a particular site (Dando et al., 2008). Shorter species are expected to live in mud volcanoes with higher concentrations of reduced compounds in upper layers of the sediment and vice-versa. Larger frenulates with long bodies are expected to bridge wider redox

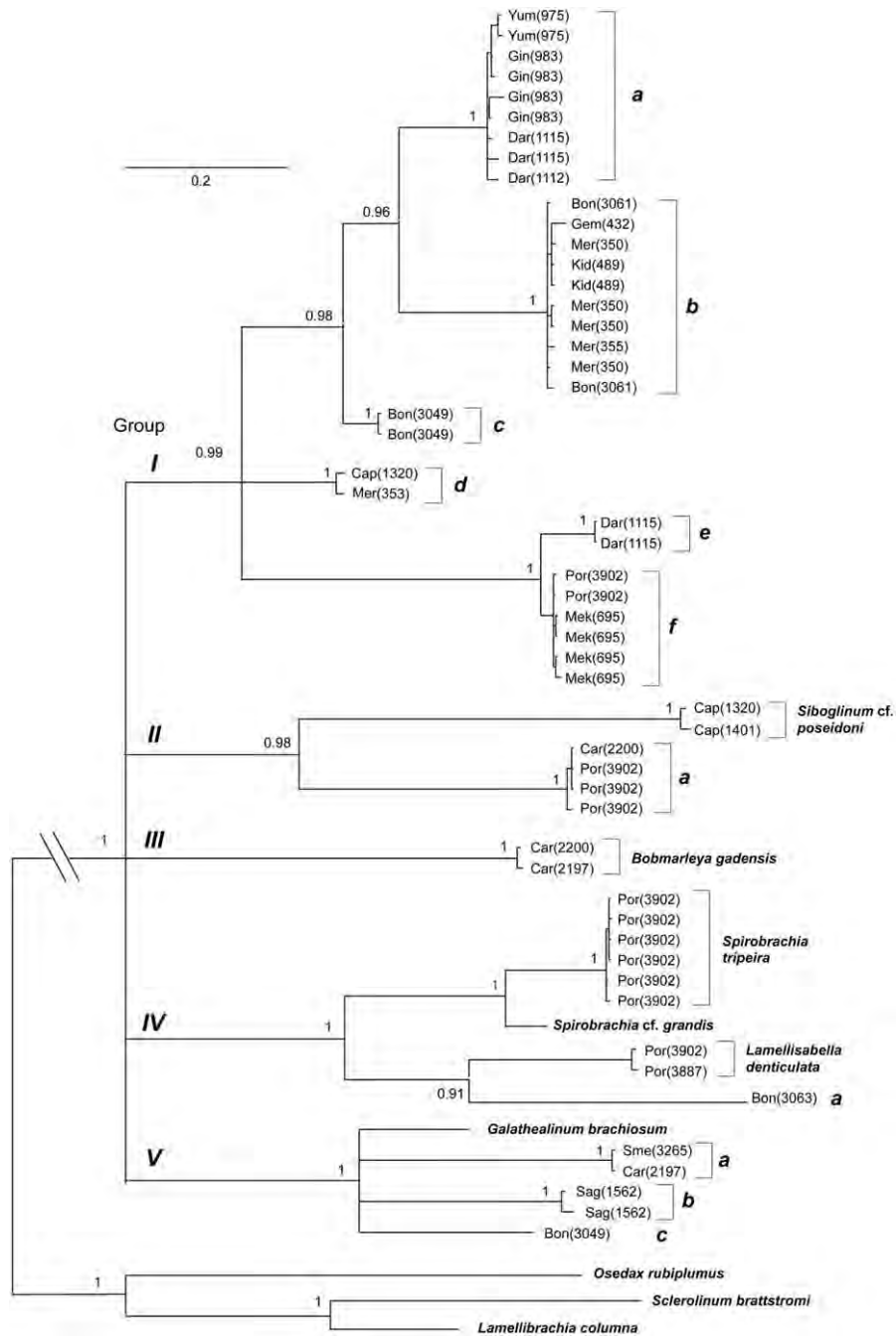


Fig. 2. Bayesian phylogenetic analysis of mitochondrial COI sequences from frenulate specimens sampled from Gulf of Cadiz mud volcanoes.

boundaries, living in sediments where reduced compounds are expelled in seep fluids or diffused to the upper sediment layers. Nonetheless, shorter and smaller species may demand less of these nutrient gases, allowing them to exploit lower concentrations that diffuse to the upper sediment. These hypotheses might help to explain the presence of the long-bodied (> 30 cm) *Lamellisabella*, *Spirobrachia* and *Bobmarleya* in Porto, Bonjardim and Carlos Ribeiro mud volcanoes where high sulphide concentrations are deep (~50 cm) below the sediment interface (Hensen et al., 2007; Nuzzo et al., 2008). However, these mud volcanoes are the deepest presently known in the Gulf of Cadiz suggesting that bathymetry may also play a role in the distribution of these species.

#### 4.3. Frenulata diversity and distribution

Frenulata is the most species-rich siboglinid clade. Since the first record in 1914 (Caullery, 1914) more than 140 species have been described. Now that frenulates are better known, these worms have been found in collections of marine benthos all over the world (Southward, 1963). Russian investigations in the western Pacific in the 1950s led to the description of a great number of species (Ivanov, 1963) and to an apparent higher diversity in the Pacific compared to other oceans. However, an increased effort put into deep-sea sampling in both sides of the Atlantic is gradually disclosing more genera as well as more species of frenulates, reducing the supposed deficiency of genera

in the Atlantic compared with the Pacific (Southward, 1978). The high diversity found in the Gulf of Cadiz, which until recently was a blank area in the distribution maps of frenulates, is an example of how important it is to explore these poorly known regions to fully understand the distribution patterns of frenulates and to draw a more complete list of species diversity, which we are still far from accomplishing.

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