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

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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

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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 μl reactions that included 30–100 ng of template DNA, 2.5 μl of ×1 of PCR buffer (supplied by manufacturer), 2.5 μl of 2.5 μM $MgCl_2$, 1 μl of each primer (10 mM final conc.), 2.5 units Amplitaq Gold DNA® polymerase (Applied Biosystems, Foster City, CA), 2.5 μl 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 μl 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 5×10^7 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.

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