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Large expansion segments in 18S rDNA support a new sponge clade (Class Demospongiae, Order Haplosclerida)

Niamh E. Redmond, Grace P. McCormack *

Molecular Evolution and Systematics Laboratory, MRI, Zoology Department, National University of Ireland, Galway, Ireland

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Abstract

Newly emerging molecular phylogenetic hypotheses involving the sponge Order Haplosclerida (Class Demospongiae) are far removed from traditional views on their classification using morphology. In the new grouping of marine haplosclerid taxa by molecular data all members of one highly supported clade were found to have three large indels in the 18S rRNA gene. These indels were not found in this gene in other marine haplosclerids or in any other demosponges analysed. These indels were found in the variable V4 and V7 region of the gene, had high GC contents and formed stable double stranded helices in the 18S rRNA secondary structure. These indels are very important synapomorphies, provide high support for an alternative taxonomic scheme and could help resolve the phylogeny of this order in conjunction with other phylogenetically informative characters.

Keywords: Porifera; Order Haplosclerida; 18S rRNA; Indels; Synapomorphies; Secondary structure; GC content

1. Introduction

Skeletal architecture and the pattern and diversity of spicules have provided most of the morphological characters in sponge systematics, including arrangement of megascleres and spongin, and the types and location of microscleres. This appears to be a successful approach for some sponge groups e.g. Tetractinellida and Calcispongia (Chombard et al., 1998; Manuel et al., 2003) but has been more problematical for others e.g. orders Halichondrida and Haplosclerida, family Axinellidae, the genus *Aplysina* (Chombard and Boury-Esnault 1999; Alvarez et al. 2000; McCormack et al., 2002; Schmitt et al., 2005; Redmond et al., 2007). Molecular biology has provided much needed insight into the evolutionary relationships of some sponge groups, particularly where morphological characteristics are few. The order Haplosclerida belongs to the Demospongiae *sensu stricto* (Borchiellini et al., 2004) and contains a very high diversity of sponges in terms of habitat and species (van Soest and Hooper, 2002).

This order is characterized by a skeleton composed of diactinal spicules which form a triangular framework in 3-dimensions. This is produced when the ends of single spicules (usually short oxeas or strongyles) are joined together by an accumulation of spongin (Hooper, 2000). Unfortunately, the haplosclerid skeleton is also characterised by a paucity of distinguishing characteristics and as a result the taxonomic history of relationships within the order is one of confusion. Originally, two groups of marine haplosclerids were divided into chalinid and renierid sponges based on the presence or absence of spongin in the skeleton respectively (Schmidt, 1870). These were then amalgamated into one group (Topsent, 1928) before Bergquist (1980) proposed two orders, the Haplosclerida and Nepheliospongida, based on reproductive and biochemical strategies even though they more or less covered the chalinid vs. renierid lines. In *Systema Porifera*, the most recent classification of the sponges, these two orders have been placed into one order Haplosclerida because of their shared unique chemistry (van Soest and Braekman, 1999) and similar spicule size

* Corresponding author. Fax: +35391 525005/+35391 494526.
E-mail address: grace.mccormack@nuigalway.ie (G.P. McCormack).

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and form (van Soest and Hooper, 2002) where they now occupy the position of suborders (Haplosclerina and Petrosina). The third haplosclerid suborder (Suborder Spongillina) contains all the freshwater sponges. Recent molecular studies using 28S rRNA, 18S rRNA, and mitochondrial CO1 gene data, showed the order to be polyphyletic with freshwater sponges more closely related to a number of other Demosponge orders (Borchiellini et al., 2004; Nichols, 2005; Redmond et al., 2007; Raleigh et al., 2007). These studies also demonstrated that members of the two recognized marine suborders, the Haplosclerina and the Petrosina, were interspersed in a number of smaller clades. Within one such clade all species were found to have unusually long 18S rRNA gene sequences and lengthy sections of the sequence had to be removed for phylogenetic analysis due to their variability (Redmond et al., 2007).

The 18S rRNA gene is approximately 1800 nucleotides long in eukaryotes (Hillis and Dixon, 1991) but unusually long sequences have been reported for a variety of distantly related organisms including protists, plathyhelminthes, and arthropods (e.g. Chalwatzis et al., 1995; Picón et al., 1996; Liu et al., 1997; Crease and Colbourne, 1998; Giribet and Wheeler, 2001; Busse and Preisfeld, 2002; Gillespie et al., 2005). The increase in length is due to large insertions in the variable regions of the gene, with the V4 and V7 regions being the most commonly affected. Variable regions (named 'V' regions) of the 18S rRNA may not be functionally important and as a result have few structural constraints. Due to this they have a higher substitution rate and thus there is greater variation between species. In the tertiary structure of the the 18S rRNA gene, conserved regions ('C' regions) are found in the centre of the ribosome and the 'V' regions are positioned on the surface (Doudna and Rath, 2002; Wuyts et al., 2001). The presence of repeated copies and the different rates of evolution among the various regions make the 18S rRNA gene quite versatile in animal systematics (e.g. Field et al., 1988; Halanych, 1995; Aguinaldo et al., 1997; Borchiellini et al., 2001, 2004; Redmond et al., 2007). The purpose of this study was to carry out a detailed investigation of the expanded variable regions in marine haplosclerids in an attempt to understand their origin and evolution. We believe that these indels are strong synapomorphies for a monophyletic group of haplosclerids (hereafter referred to as Clade X) not previously described in morphological classifications, and may help resolve the phylogeny of the order in conjunction with other phylogenetically informative characters. The use of new molecular synapomorphies may help provide answers to the many questions in both haplosclerid and sponge systematics as a whole.

2. Materials and methods

2.1. Alignments and identification of secondary structure elements and variable regions

A multiple alignment of all available sponge 18S rRNA sequences was assembled in MacClade 4.0 (116 sequences retrieved from GenBank including 22 marine haplosclerid sequences, 20 of which were generated by the authors previously) to make a search for expansion segments in this gene across the taxon. An alignment was subsequently created, which contained complete sequences from marine haplosclerids ($n = 22$) only and was a total of 2065 nt long. Only partial 18S rRNA sequences could be produced from seven additional haplsoclerid species (*Amphimedon viridis, Amphimedon compressa, Gelliodes fibulata, Haliclona vanderlandi, Hemigellius rudis, Neopetrosia* sp. and *Strongylophora* sp.). These partial sequences were employed to investigate the presence of indels, but as they were incomplete they were excluded from the phylogenetic analysis. To define the core secondary structure elements for the entire 18S rRNA molecule of the various sponge taxa, our primary sequences were compared to the eukaryotic 18S rRNA variability map (Wuyts et al., 2002) and three previously published secondary structure models of *Drosophila melanogaster*, *Daphnia pulex*, and *Toxoplasma gondii* (Van de Peer et al., 1998; Crease and Colbourne, 1998; Gagnon et al., 1996, respectively). A putative secondary structure model for the *Haliclona cinerea* sequence was manually prepared. MFold version 2.3 (Zuker, 2003; Walter et al., 1994) was employed to suggest stable secondary structures for the areas that could not be easily defined (e.g. expansion segments and helices 10, 10e−1 (both in the V2 region), 12-1 and 45/46 (V8 region)). The default settings for all parameters were used except for the folding temperature, which was set at 20 °C for all haplosclerid sequences due to the ambient temperature where these sponges occur (Wörheide et al., 2004). Where multiple foldings were returned the structure with the lowest free energy was chosen. The nomenclature of the secondary structure was implemented as outlined in Wuyts et al. (2002). The program RnaViz2 was used to draw the secondary structures (De Rijk et al., 2003). The V4 region of the haplosclerid sequences was defined as the area between helices 23 and 24 and the V7 region was defined as covering helix 43 (Wuyts et al., 2000) and alignments of both these regions are available in Supplementary Material (Parts 1 and 2, respectively) with the associated indels highlighted.

2.2. Phylogenetic analysis

Phylogenetic trees were reconstructed from complete 18S rRNA sequences of marine haplosclerid taxa with the three indel regions both included and excluded. For tree drawing purposes a conservative alignment strategy was employed in each case, where all ambiguously aligned positions were excluded prior to analyses. The phylogeny shown in Redmond et al. (2007) indicated that the sister group to Clade X contained *Acanthostrongylophora ingens*, *Petrosia* sp. A and sp. B, *Chalinula hooperi* and *Niphates* sp. These were therefore employed as outgroups to the remaining taxa. An appropriate model of evolution (TrN+I+G model with base frequencies A=0.24, C=0.21, G=0.30, T=0.25; R(A-G)=0.272, R(C-T)=5.7,