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Polyphyly of the genus *Axinella* and of the family Axinellidae (Porifera: *Demospongiae*[p])

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ABSTRACT

The genus *Axinella* is difficult to define on the basis of morphological characters and includes a heterogeneous assemblage of species. Several previous authors have suspected the polyphyly of both this genus and the family Axinellidae. To clarify the phylogeny of Axinellidae and *Axinella*, we propose a new hypothesis based on two molecular markers.

In our analyses, Axinellidae and *Axinella* are polyphyletic assemblages. The 15 species of Axinellidae in our dataset belong to five clades and the nine species of *Axinella* to three clades. One *Axinella* clade, named *Axinella*[p], contains the type-species of the genus: *A. polypoides* (plus *A. aruensis, A. dissimilis, A. infundibuliformis* and *A. vaceleti*). A new clade, *Cymbaxinella*[p], is proposed, following the *PhyloCode*, it includes *C. damicornis, C. verrucosa, C. corrugata* and *C. cantharella*. The species *Axinella cannabina* is reallocated to a clade named *Acanthella*[p]. The clades *Agelas*[p] and *Cymbaxinella*[p] constitute a new clade: *Agelasida*[p]. Few morphological, biochemical and secondary structures characters support these groupings, highlighting the need for new characters for such problematic sponge groups. This work is an attempt to build a framework for the phylogeny of taxa allocated to *Axinella* and Axinellidae in the traditional classification.

1. Introduction

The phylogeny of *Demospongiae*[p][1] has been revisited recently and congruent results have been obtained with ribosomal DNA (Borchiellini et al., 2004), mitochondrial DNA (Lavrov et al., 2008) and nuclear housekeeping genes (Sperling et al., 2009). Four clades, G1 (*Keratosa*[p]), G2 (*Myxospongiae*[p]), G3 (marine Haplosclerida) and G4 (Spongillina, *Tetractinellida*[p], Poecilosclerida, Agelasida, Halichondrida, Hadromerida), have been defined (Borchiellini et al., 2004). *Tetractinellida*[p] is a well supported sub-clade (see Erpenbeck and Wörheide, 2007 for references), but the other orders included in G4 are not found again. All these orders, as well as many families and genera, seem to be polyphyletic in all the phylogenetic works so far and the phylogenetic relationships within G4 have not been elucidated (Erpenbeck and Wörheide, 2007).

As underlined by Cárdenas et al. (2010) "Sponge molecular phylogenies are becoming more common and diversified every year but, unfortunately, many of these are not translated into classifications thus accentuating the phylogeny classification gap and the creation of 'phantom taxonomies'". Many recent molecular phylogenies (Borchiellini et al., 2004; Erpenbeck et al., 2004, 2005, 2007c; Nichols, 2005; Erpenbeck and Wörheide, 2007; Lavrov et al., 2008) have suspected the polyphyly of genera, families and order. The sponge classification system is based essentially on the characteristic features of the skeleton, and the shape and size of its constitutive elements (i.e., the spicules and the spongin fibres). However, spongologists are faced with a huge problem, i.e., the absence of knowledge about the homology of characters and the ancestral state of a character (Bergquist and Fromont, 1988; Fromont and Bergquist, 1990; Boury-Esnault, 2006). Consequently, hypotheses regarding primary homology are established on exceedingly weak grounds. In spicule nomenclature, one name corresponds to a general form and is only descriptive without being indicative of homology (Fromont and Bergquist, 1990). A good example of this difficulty of resolution is *Axinella* Schmidt, 1862, type genus of the family Axinellidae Carter, 1875 (Solé-Cava and Boury-Esnault, 1999; Alvarez et al., 2000; Borchiellini et al., 2004; Erpenbeck et al., 2004, 2005, 2007a; Nichols, 2005; Erpenbeck and Wörheide, 2007; Lavrov et al., 2008; Redmond and McCormack, 2008). It is particularly important to resolve this question, at a time where more and more works on natural products deal with Axinellidae species (Braekman et al., 1992; Aiello et al., 2006, 2007; Vergne et al., 2006, 2008; Costantino et al., 2008; Gabant et al., 2009a, 2009b).

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[1] [p]Following the *PhyloCode*, the name of clades are in italics with [p] (Art 6.1A and 6.1B).

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The genus *Axinella* Schmidt, 1862 currently includes a heterogeneous assemblage of species although a recent definition of *Axinella* is given in Systema Porifera (Alvarez and Hooper, 2002): "Axinellidae with choanosomal skeleton differentiated in axial (condensed or vaguely reticulated) and extra axial (plumoreticulated) region. Megascleres are styles or oxeas. Microscleres if present are microraphids and trichodragmata". Many species included in *Axinella* lack some of the features which are generally recognised as typical of the genus. In addition, some of the characters can be variable at the intra-specific level. Ridley and Dendy (1887, p. 178) have underlined that *Axinella* "is a very critical genus, and it is impossible to give a satisfactory diagnosis of it". This problem of giving a satisfactory diagnosis of this genus has, to date, made little progress and sheds doubt on the real phylogenetic status of this group (Alvarez et al., 2000; Alvarez and Hooper, 2002).

The most recent definition of the Axinellidae is given by Alvarez and Hooper (2002): Halichondrida without specialised ectosomal skeleton; with velvety or microhispid surface; with choanosomal skeleton of ascending spiculo-fibres connected irregularly by loose spicules and short tracts, or plumoreticulated with ascending plumose tracts connected by thinner ones or single spicules. Megascleres are oxeas, anisoxeas, styles, sinuous strongyles in any combination. Microscleres are raphides, single or in trichodragmata (Alvarez et al., 2000; for a complete history of the family see Alvarez and Hooper, 2002, pp. 724–726).

These subjective characters led in the last fifty years to two main opinions. Lévi (1973) has erected an order Axinellida for the family Axinellidae and other close families such as Bubaridae, Desmoxyidae, Hemiasterellidae, Trachycladidae, Raspailiidae, Euryponidae, Rhabderemiidae, Sigmaxinellidae (see Supplementary file 1) based on the characters of the skeleton and the type of reproduction (oviparity). Alvarez and Hooper (2002) following Soest et al. (1990) abandoned the order Axinellida and reattributed the families to Poecilosclerida (Raspailiidae, Euryponidae, Sigmaxinellidae, Rhabderemiidae), Hadromerida (Hemiasterellidae, Trachycladidae) and included the Axinellidae (with Bubaridae and Desmoxyidae) among the Halichondrida. The data taken into account in this classification were mainly based on the type of megascleres and microscleres present in the different genera and families. The recent molecular phylogenies have never recovered the monophyly of an order Halichondrida (Erpenbeck et al., 2004; Erpenbeck and Wörheide, 2007).

In order to clarify the phylogeny of Axinellidae and *Axinella*, a new hypothesis based on two independent molecular markers (18S and 28S rRNA) and predicted secondary structures features, that have been shown to increase the accuracy of phylogenetic reconstruction (Voigt et al., 2008) and that can be considered as molecular synapomorphies is proposed. An appropriate sample of *Axinella* (9 species) and three other genera of the family Axinellidae and close families Dictyonellidae (3 genera), Desmoxyidae (1 genus), Agelasidae (5 species), Halichondriidae (1 genus), Suberitidae (2 genus) and three outgroups belonging to *Tetractinellida*^{p}, was used to address this issue (Table 1).

2. Materials and methods

2.1. Sampling and sequence acquisition

The species analysed in this study and their collecting sites are listed in Table 1 (new sequences are indicated in bold characters). Procedures used for genomic DNA extraction, cloning and DNA sequencing are described in Borchiellini et al. (2001). For partial 28S rDNA amplification (400–450 bp) and for total 18S rDNA amplification, the primers used are the same as those described previously (Borchiellini et al., 2001). For 28S rDNA, another set of primers was designed to obtain a longer fragment (1100–1200 bp) containing domains C1 to D3: Forward (S1) ACC CGC TGA ATT TAA GCA T, Reverse (Y) AGT CTT TCG CCC CTA TAC CCA. The total 18S and partial 28S rDNA gene sequences resulting from this work have been deposited in the GenBank database. Their respective accession numbers are listed in Table 1. Others sequences used for this work came from GenBank (Table 1).

2.2. Sequence alignment and phylogenetic analysis

Initial sequence alignment was performed using the online software Muscle (http://www.ebi.ac.uk/Tools/muscle/index.html) (Edgar, 2004a,b), then subsequently corrected by eye on Bioedit Sequence Alignment Editor 5.09 (Hall, 1999). Ambiguously aligned regions were determined by the programme Gblocks version 0.91 b (Castresana, 2000), and excluded from the analyses. After trying a variety of settings, the final Gblocks settings were selected to yield a good quality alignment while not sacrificing an unnecessarily large amount of data. A removal of 7%, and 24% of the 18S and 28S alignments, respectively, was thus performed. The settings were as follows for both the 18S rRNA and 28S rRNA partitions: [1:17; 2:17; 3:8; 4:4; 5:with half]. The character exclusion sets based on Gblocks are available upon request through the corresponding author.

The final 18S alignment contains 33 species, 1692 characters, of which 1397 are constant, 74 are parsimony uninformative and 221 parsimony informative. The final 28S alignment contains 33 species, 931 positions, of which 443 are constant, 132 are parsimony uninformative and 356 parsimony informative. A combined dataset was created by adding 18S and 28S rDNA sequences for the same set of organisms whenever possible (29 species). This combined dataset contains 2623 positions, 1853 of which are constant, 238 parsimony uninformative and 532 parsimony informative. The alignments have been deposited on the free TreeBASE database (numbers SN4967, SN4968, SN4969) for 18S, 28S and 18S + 28S, respectively (www.treebase.org) (Sanderson et al., 1994; Morell, 1996).

Phylogenetic analyses were performed using parsimony, Maximum Likelihood (ML) and Bayesian methods. We used PAUP 4.0 (Swofford, 2000) for Maximum Parsimony (MP) analyses. The characters were always treated as unordered and equally weighted (contrary to Bayesian analyses). MP trees were computed using heuristic searches with 100 replicates of random taxon addition sequence and TBR branch swapping. For ML analyses we used MrModeltest (modified version of ModelTest 3.6) (Posada and Crandall, 1998; Nylander, 2004) to determine the best-fitting nucleotide substitution model for each data partition, excluding ambiguously aligned regions from the calculations. This resulted in the following models being used for 18S, 28S and 18S + 28S, respectively: GTR + G + I (general time-reversible model + gamma distributed rates of substitution + estimated proportions of invariant sites), GTR + G and GTR + G + I. Then, we performed the analyses with the PhyML software (Guindon and Gascuel, 2003). Parameters were set using MrModeltest as follows for 18S, 28S and 18S + 28S, respectively: (I = 0.6445, gamma distribution shape parameter = 0.7852; I = 0, Gamma distribution shape parameter = 0.4098; I = 0.4788, gamma distribution shape parameter = 0.5654). Between site variation was estimated using a discrete approximation of the gamma distribution with 4 rate categories. Gaps were treated as missing data. The statistical robustness of the tree topology was assessed by bootstrap resampling (1000 replicates for MP and ML).

In molecules constrained by secondary structure such as 18S and 28S rDNA, the nucleotides involved in stems and loops do not evolve independently, as assumed with standard models of substitution such as those compared in (MrModeltest) (Wheeler and Honeycutt, 1988; Dixon and Hillis, 1993). Mixed models of