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## Mitochondrial Genome of *Savalia savaglia* (Cnidaria, Hexacorallia) and Early Metazoan Phylogeny

F. Sinniger,<sup>1,2</sup> P. Chevaldonné,<sup>2</sup> J. Pawlowski<sup>1</sup>

<sup>1</sup> Department of Zoology and Animal Biology, Molecular Systematic Group, Science III, University of Geneva, 30 quai Ernest-Ansermet, 1211 Genève 4, Switzerland

<sup>2</sup> Centre d'Océanologie de Marseille, UMR CNRS 6540 DIMAR, Université de la Méditerranée, Station Marine d'Endoume, rue de la Batterie des Lions, 13007 Marseille, France

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**Abstract.** Mitochondrial genomes have recently become widely used in animal phylogeny, mainly to infer the relationships between vertebrates and other bilaterians. However, only 11 of 723 complete mitochondrial genomes available in the public databases are of early metazoans, including cnidarians (Anthozoa, mainly Scleractinia) and sponges. Although some cnidarians (Medusozoa) are known to possess atypical linear mitochondrial DNA, the anthozoan mitochondrial genome is circular and its organization is similar to that of other metazoans. Because the phylogenetic relationships among Anthozoa as well as their relation to other early metazoans still need to be clarified, we tested whether sequencing the complete mitochondrial genome of *Savalia savaglia*, an anthozoan belonging to the order Zoantharia (=Zoanthidea), could be useful to infer such relationships. Compared to other anthozoans, *S. savaglia*'s genome is unusually long (20,766 bp) due to the presence of several noncoding intergenic regions (3691 bp). The genome contains all 13 protein coding genes commonly found in metazoans, but like other Anthozoa it lacks most of the tRNAs. Phylogenetic analyses of *S. savaglia* mitochondrial sequences show Zoantharia branching closely to other Hexacorallia, either as a sister group to Actiniaria or as a sister group to Actiniaria and Scleractinia. The close rela-

tionships suggested between Zoantharia and Actiniaria are reinforced by strong similarities in their gene order and the presence of similar introns in the COI and ND5 genes. Our study suggests that mitochondrial genomes can be a source of potentially valuable information on the phylogeny of Hexacorallia and may provide new insights into the evolution of early metazoans.

**Key words:** Mitochondrial genome — Early metazoan — Cnidaria — Anthozoa — Hexacorallia — Zoantharia

### Introduction

Mitochondrial genomes are commonly used to infer metazoan phylogeny, their haploid status and their small size being the most important advantages as phylogenetic markers. Seven hundred twenty-three complete mitochondrial genomes are available in the public databases (GenBank, January 2006) and this number is rapidly increasing. DNA sequences of tRNA, rRNA, and protein coding genes and inferred amino acid sequences of protein coding genes as well as gene order provide different types of information useful at different taxonomic levels (Boore 1999). If mitochondrial DNA sequences are widely used for species identification (Herbert et al. 2004; Ward et al.

Correspondence to: F. Sinniger; email: frederic.sinniger@zoo.unige.ch

2005) and for inferring relationships below the family level, rearrangements in gene order are mainly informative for relationships between phyla (Jennings and Halanych 2005). However, such rearrangements have in some cases also been used to infer phylogeny within phyla (Dreyer and Steiner 2004) and even within families (Mabuchi et al. 2004).

Among the metazoan mitochondrial genomes sequenced up until now, only 11 come from diploblastic organisms. Three of these mitochondrial genomes belong to demosponges: *Tethya actinia*, *Geodia neptuni* (Lavrov et al. 2005), and *Axinella corrugata* (Lavrov and Lang 2005). The other genomes belong to anthozoans (Cnidaria), including the soft coral *Sarcophyton glaucum* (Octocorallia, Alcyonaria) (Beaton et al. 1998), the sea anemone *Metridium senile* (Hexacorallia, Actiniaria) (Beagley et al. 1998), and the six hard corals (Hexacorallia, Scleractinia). The scleractinian genomes include three species belonging to the family Acroporidae—*Acropora tenuis* (Van Oppen et al. 2002), *Anacropora matthai*, and *Montipora cactus* (Tseng et al. 2005)—and three others belonging to the *Montastrea annularis* species complex (Fukami and Knowlton 2005). In addition to completely sequenced mitochondrial genomes, there is also information on the order of genes in the mitochondrial genome of *Renilla kollikeri* (Octocorallia, Pennatulacea) (Beagley et al. 1995) and regarding the organization of the genome in *Acropora nasuta* (Fukami et al. 2000). All these data show that, in opposition to Hydrozoa and other Medusozoa, which possess linear mitochondrial genomes in one or more parts (Bridge et al. 1992), all anthozoans sequenced so far have circular mitochondrial genomes like other metazoans.

The acquisition of the first complete mitochondrial genomes of cnidarians and sponges prompted investigation of the utility of their use in inferring early metazoan phylogeny. Until recently, the phylogenetic study of the diploblastic taxa at higher taxonomic levels was based mostly on the sequences of 28S and 18S rDNA (Medina et al. 2001; Collins 2002; Borchellini et al. 2004). Despite such studies, different phylogenetic questions concerning the relationships between diploblasts and bilaterians and relationships within different diploblastic taxa remain partially or completely unanswered. Relationships at the base of the metazoan tree have proven to be difficult to resolve even with multigene analyses of nuclear DNA (Rokas et al. 2003, 2005).

Mitochondrial genes have mostly been used to address phylogenetic questions at lower taxonomic levels (Shearer et al. 2002). COI has by far been the most widely used mitochondrial marker, but its resolution is very variable among taxa (Fukami et al. 2004; Addis and Peterson 2005; Nichols and Barnes 2005). The two mitochondrial rDNA subunits have

been extensively used in cnidarian phylogeny but do not provide much information at the species level (Romano and Palumbi 1997; Sanchez et al. 2003; Sinniger et al. 2005). Among Octocorallia the efficiency of mitochondrial markers is limited to the genus level (McFadden et al. 2004) despite *msh1* and some ND subunits being more variable and useful in solving phylogeny within suborders (Sanchez et al. 2004).

To increase taxon sampling of cnidarian mitochondrial genomes and to test their possible interest for phylogenetic use, here we have sequenced the complete mitochondrial genome of *Savalia savaglia*, a species belonging to the hexacorallian order Zoantharia (= Zoanthidea, Zoanthiniaria). Phylogenetic analyses of obtained sequences confirm the monophyly of Hexacorallia and give insights into the relationships among three orders (of a total of six) (Zoantharia, Actiniaria, Scleractinia) belonging to this class. In view of our data, the gene rearrangements in mitochondrial genomes provide important phylogenetic information at this taxonomic level.

## Materials and Methods

### Sampling and DNA Extraction

*Savalia savaglia* (formerly in the genus *Gerardia*) was collected from a colony originating from the Embiez Islands (France) and artificially implanted and reproduced on a small island off Marseille (France). It is the only zoantharian (=zoanthid) genus to secrete its own skeleton covering the substrate (usually gorgonians or antipatharians). *S. savaglia* is found in the Mediterranean Sea at depths ranging from 30 to more than 100 m. The tissues were fixed and conserved in 70% ethanol and two DNA extractions were performed using the DNeasy Plant Minikit (Qiagen). To avoid contamination from epizoid organisms, DNA was extracted from a fragment of mesenteria carefully isolated under the microscope. Genomic DNA obtained was used to perform PCR amplifications. The absence of contamination was confirmed by sequence analyses, as none of the sequences obtained were similar to any potential prey, parasite, epibiont, or other cnidarian species present in the area. Moreover, the overlap between new sequences and previously obtained zoantharian sequences (COI, 12S, 16S) warranted the homogeneity of genomic material.

### PCR Amplification and Sequencing

PCR conditions were as described by Sinniger et al. (2005). PCR primers were designed from the alignment of the three anthozoan mitochondrial genomes available at the time (*S. glaucum*, *A. tenuis*, *M. senile*) to complement the universal primers HCO2198, LCO1490 (Folmer et al. 1994), BB5, 16Sar, and 16Sbr (Palumbi et al. 1996). The primer sequences and position on the genome are available as supplementary material. Not knowing the order of the genes, all different combinations between primers were tested. This method allowed us to obtain a 13,500-bp contig. The remaining 7200 bp was amplified using long-range PCR. LCO and BB5 universal primers (Palumbi et al. 1996) were used to amplify the COI intron for different closely related species. Sequencing was made by primer-walking. Direct sequencing was carried out using a BigDye

Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequences were performed on an ABI-3100 Avant automatic sequencer. The complete sequence of the *S. savaglia* mitochondrial genome is available in GenBank (DQ825686).

### Sequence Analyses

The sequences were edited, aligned, and concatenated using the BIOEDIT program (Hall 1999). Transfer RNA genes were searched using the tRNAscan-SE program (Lowe and Eddy 1997) protein genes were first identified using the similarity search in NCBI GenBank using the BLAST network service (Benson et al. 2003) and then compared with the corresponding aligned genes of other anthozoans.

Two sets of sequences were analyzed. The first set consisted of an alignment kindly provided by D. Lavrov as used by Lavrov et al. (2005), to which we added the sequence of *S. savaglia*. The second set was created to increase the number of metazoans considered in the analyses, it included 18 metazoans and the choanoflagellate *Monosiga brevicollis* as an outgroup (accession numbers available in the Supplementary Material). Hydrozoans were not included in the analyses due to their high divergent evolutionary rates.

The first alignment (from Lavrov et al. 2005) was unmodified after the addition of *S. savaglia* and analyzed with Bayesian inference (BI) and maximum likelihood (ML) methods using the MrBayes (Ronquist and Huelsenbeck 2003) and PHYML programs, respectively. For Bayesian analyses we ran four Markov chain Monte Carlo chains for 200,000 generations, using the mtREV matrix of amino acid substitutions, a gamma 1 invariant, and 12 categories. Trees were sampled every 10th cycle after the first 10,000 burn-in cycles. For ML analyses, we used the PHYML program (Guindon and Gascuel 2003) with the mtREV matrix of amino acid substitutions, a gamma 1 invariant model with eight categories, estimated  $\alpha$ -parameter, and estimated frequencies of amino acids. Bootstrap was performed generating hundred replicates with the SEQBOOT program in the PHYLIP package (Felsenstein 2002) and analyzing them as previously mentioned.

For the second set of data, alignments were built using ClustalW 1.82 (Thompson et al. 1994) with different opening-extension gap penalties and compared using the SOAP program (Löytynoja and Milinkovitch 2001) according to Lavrov et al. (2005). However, artifactual results with a few clearly nonhomologous sites were obtained using SOAP, and such sites were manually deleted from the alignment. Phylogenetic analyses were performed for each gene independently using the ML method as described above. Homologous sites in different genes were concatenated to obtain an alignment of 2458 sites. This alignment was analyzed using BI and ML as previously described. The same analyses were conducted on an alignment containing 3500 sites consisting of only diploblastic representatives and *Monosiga brevicollis* as an outgroup. Relative rate tests were made using the Dambe program (Xia and Xie 2001).

We also used the GRIMM program (Tesler 2002) to analyze gene rearrangement within the mitochondrial genomes. However, because this program requires identical genome contents, its use in our case was limited. We used it to analyze gene order within anthozoans (ignoring the presence of MutS in *Sarcophyton* and tRNA<sup>W</sup> in *Metridium* and *Acropora*).

## Results and Discussion

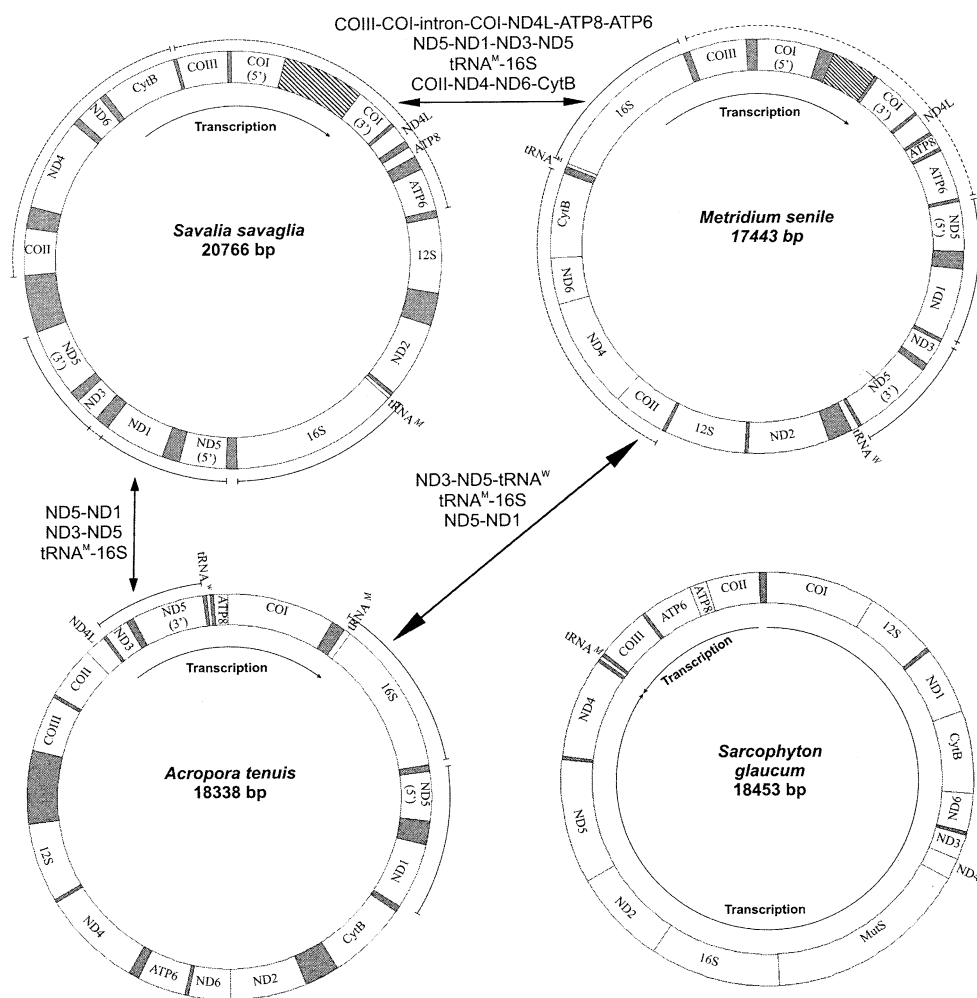
### Genome Characteristics

The most striking feature of the mitochondrial genome of *S. savaglia* is its unusual length. Compared to

other sequenced anthozoans, whose mitochondrial genome sizes range between 16,137 bp in *Montastrea franksi* (Fukami and Knowlton 2005) and 18,453 bp in *S. glaucum* (Beaton et al. 1998), the mitochondrial genome of *S. savaglia* is 20,766 bp long. Among early metazoans, only the sponge *Axinella corrugata* possesses a longer (25,610-bp) mitochondrial genome (Lavrov and Lang 2005).

The unusual length of the *S. savaglia* mitochondrial genome is due primarily to the presence of long noncoding intergenic regions (3691 bp). On average, there is 231 bp of noncoding DNA per gene in *S. savaglia*. This number is significantly higher than in other anthozoans, in which the length of noncoding mitochondrial DNA per gene ranges from 26 bp in the octocorallian *S. glaucum* to 124 bp in the actiniarian *M. senile* and 172 bp in the scleractinian *A. tenuis*. The noncoding mitochondrial DNA in *S. savaglia* is not homogeneously distributed between the genes, but instead there are two major intergenic regions. The first major region (934 bp) is located between ND5 and COII (Fig. 1), whereas the second major region (518 bp) is situated between 12S and ND2. The remaining 2239 bp of noncoding data is distributed between the other genes in fragments between 20 bp (tMet-16S) and 333 bp (COII-ND4) long.

The mitochondrial genome of *S. savaglia* contains all 13 protein-coding genes commonly found in metazoan mitochondrial genomes (ATP6, ATP8, COI, COII, COIII, Cytb, ND1, ND2, ND3, ND4, ND4L, ND5, and ND6), two rRNA subunits (12S and 16S), and only one tRNA (tRNA<sup>M</sup>) (Fig. 1). Like all other anthozoan mitochondrial genomes sequenced up until now, the genome of *S. savaglia* lacks most of the tRNAs. However, it differs from other anthozoans by possessing only the tRNA for methionine, while scleractinians and *M. senile* also possess the tRNA for tryptophane (tRNA<sup>W</sup>). The absence of tRNA genes is not typical for most early metazoans. The Demospongiae possess not only all the usual genes commonly found in metazoan mitochondrial genomes but also some additional tRNAs as well as the ATP9 gene (Lavrov et al. 2005). Additional mitochondrial genes have also been reported in Octocorallia, which share a supplementary gene coding for a protein similar to the bacterial MutS (Sanchez et al. 2003; unpublished data). There is a general trend toward larger sizes of mitochondrial genomes in diploblastic animals and reduced sizes in bilateria, with the exception of a few mollusks and amphibians (Sano et al. 2005; Mueller et al. 2004). Different taxa show increases in the size of their mitochondrial genome due to either the presence of long noncoding regions, as shown in *S. savaglia*, or to the presence of additional genes.



**Fig. 1.** Schematic plans of four anthozoan mitochondrial genomes, the Hexacorallia *S. savaglia* (Zoantharia), *M. senile* (Actiniaria), and *A. tenuis* (Scleractinia) and the Octocorallia *S. glaucum* (Alcyonacea). Solid lines around the genomes indicate conserved clusters among the three Hexacorallia; dashed lines indicate clusters shared only between *S. savaglia* and *M. senile*. Intergenic regions are represented in gray and the COI intron is represented by a hatched area. The arrows between the three hexacorallian genomes indicate the gene clusters shared between them.

Interestingly, the nucleotide composition of the mitochondrial DNA of *S. savaglia* shows a surprisingly small bias toward A + T (51.7%) compared to other anthozoan (> 60%) and, in general, metazoan (> 70%) mitochondrial genomes (Lavrov et al. 2005). The protein coding genes show a slightly higher value of A + T (54.3%) but this is still significantly lower than in other metazoans. Compared to most Bilateria, which use different genetic codes to translate mitochondrial-encoded proteins, the only deviation from the universal code observed in *S. savaglia* is the same as that found in other cnidarians, sponger, or protists, with TGA coding for Trp instead of being a stop codon. All the mitochondrial genes of *S. savaglia* are coded on the same strand as in other Hexacorallia.

#### Gene Rearrangement and Introns

The gene order in the *S. savaglia* mitochondrial genome is clearly similar with that of *M. senile*, suggesting a close relationship between Zoantharia and Actiniaria. Four clusters of genes are shared between

*S. savaglia* and *M. senile* (tRNA<sup>M</sup>-16S, ND5-ND1-ND3-ND5, COII-ND4-ND6-CytB, and COIII-COI-ND4L-ATP8-ATP6). The only cluster common to all Hexacorallia sequenced until now is the cluster grouping tRNA<sup>M</sup> and 16S. Another similarity concerns the ND5 intron, which starts with ND1 and ends with ND3 in all Hexacorallia. Moreover, Scleractinia shares the presence of the tRNA<sup>W</sup> with Actiniaria. This gene is situated after ND5 in both orders (Fig. 1). The results obtained with the GRIMM program confirmed the relationship between *Metridium* and *Savalia*. Gene order similarity between Antipatharia (*Stauropathes*) and Actiniaria, which share the segment 16S-COIII-COI, was also observed (Brugler and France 2003; unpublished data).

On the other hand, two group I introns, one present in the ND5 gene and the other in COI, are shared by actinarian and zoantharian mitochondrial genomes. The introns were identified by comparison with the introns found in other Hexacorallia and showed the same characteristics. The intron inserted in the ND5 gene is identical to the one found in

*M. senile* (Beagley et al. 1996). Both contain the genes coding for ND1 and ND3 and the insertion site is at the same position. Although this intron is also present in Scleractinia, in this order it includes almost all genes. However, the presence of ND1 at the beginning and ND3 at the end of the intron is common to all Hexacorallia. The intron in COI is located 17 bp before the one found in *M. senile*, suggesting its homoplastic origin. This difference could explain why the intron was not detected in zoantharians in previous studies (Beagley et al. 1996). As in *M. senile*, the ORF contained in this intron codes for a homing endonuclease of the LAGLI\_DADG subclass, but the two ORFs are too divergent to be alignable.

The COI intron was also found at exactly the same location in other zoantharians belonging to the same family as *S. savalia* (Parazoanthidae) (data not shown). PCR amplification suggests the presence of an intron of comparable size in the genus *Epizoanthus* (Zoantharia, Epizoanthidae) and in an unidentified black coral (Antipatharia), but no sequences were obtained to confirm this result. Moreover, the COI intron seems to be present in some scleractinians too (Fukami, unpublished data). If this is confirmed, the COI intron could be a mobile element potentially present in all Anthozoa, while the ND5 intron, comprising the ND3 and ND1 genes, could be a synapomorphy of Hexacorallia.

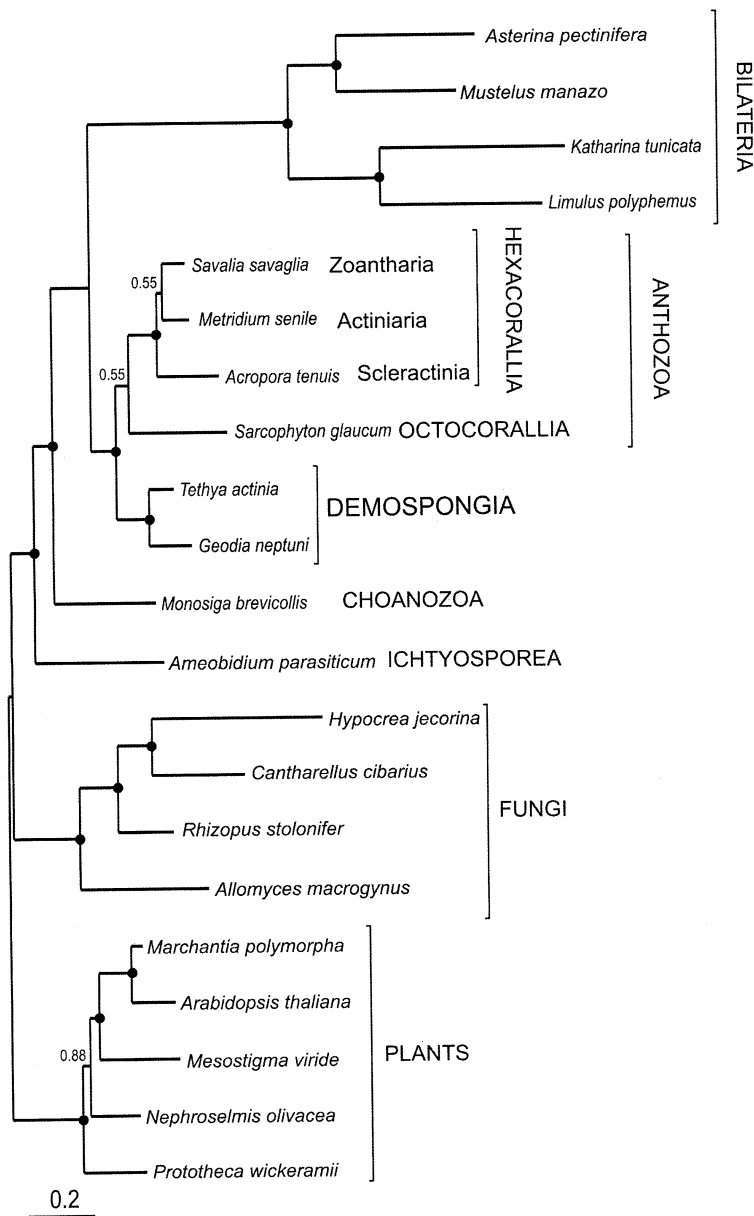
### Phylogenetic Analyses

Our two alignments analyzed comprised representatives of the major metazoan groups (sponges, cnidarians, arthropods, echinoderms, and vertebrates). While Lavrov's alignment focused more on the outgroups (plants, Fungi, and Ichtyosporae), our alignment comprised more metazoan taxa (annelids, mollusks, brachiopods, myriapods, crustaceans, and insects). Depending on the taxa selected, we found striking differences in the phylogenetic relationships among Anthozoa. The Bayesian tree (Fig. 2) obtained using Lavrov's alignment shows the monophyly of Anthozoa, albeit with very weak support (0.55). In this analysis, *S. savaglia* branches as a sister group of *M. senile*, but this clade is also weakly supported (0.55). On the other hand, in analyses of alignments including only metazoans and *Monosiga* as an outgroup (Figs. 3A and B), the Anthozoa appear as a paraphyletic group, with *S. glaucum* branching as a sister group of Demospongiae and Hexacorallia. The position of *S. glaucum* was identical whether we considered only diploblasts (Fig. 3B) or a wider sampling including bilaterians (Fig. 3A). However, the relationship between *M. senile* and *S. savaglia* was different depending on the tree. Both

species group together in the ML analysis of a smaller number of taxa, while they branch independently in the BI analysis and with both methods when bilaterian sequences are included. This may be due to the increase in the number of analyzed positions, which averages 3500 in the first case and 2458 in the second case. However, the bootstrap values and posterior probabilities for both topologies were very weak (posterior probabilities 0.56, bootstrap <0.5, and posterior probabilities 0.56, bootstrap 0.99, respectively).

To test the position of *S. glaucum* at the base of diploblasts, we analyzed each mitochondrial gene separately. Most of the single-gene analyses confirmed the unexpected position of *S. glaucum* (Table 1). Among protein-coding genes, only COII, CytB, ND3, ATP8, and ND6 support the monophyly of Anthozoa, the two last genes being too variable to include Bilateria in the alignment. However, the trees (not shown) obtained with these genes that show anthozoans as a monophyletic group were not all congruent. ATP6 shows that demosponges are paraphyletic, ND3 places them at the base while in the ND6 tree *S. glaucum* branches within Hexacorallia. The two mitochondrial ribosomal genes (12S and 16S) support the monophyly of Cnidaria, however, the 12S analysis places demosponges at the basis of Bilateria and the addition a hydrozoan sequence creates a long-branch attraction with *S. glaucum* at the basis of Bilateria.

Rather than suggesting that anthozoans are a polyphyletic group, we further tested whether the position of *S. glaucum* could be due to an increased evolutionary rate in this species. Indeed, the relative rate test showed that 11 of 15 mitochondrial genes (ATP6, ATP8, COI, COII, ND2, ND4, ND4L, ND5, ND6, 12s, and 16S) evolve at a significantly different rate in *S. glaucum*, compared to other diploblastic animals (Table 1). This could explain the artifactual long-branch attraction between this species and rapidly evolving Bilateria. However, in spite of the differences between hexacorallian and octocorallian sequences, the rates of evolution within the latter group do not seem to be particularly rapid. Indeed, the genome of *S. glaucum* (order Alcyonacea) shows the same gene order as the octocorallian *Renilla kolikeri*, which belongs to the order Pennatulacea (Beagley et al. 1995). Different phylogenetic studies made on intergenic regions among octocorallians support the conservation of the gene order among octocorallians (France and Hoover 2001, 2002; Smith et al. 2003; McFadden et al. 2004). The difference between Hexacorallia and Octocorallia could be explained by acceleration in the stem lineage of Octocorallia followed by a slowdown of rates during the radiation of the group.



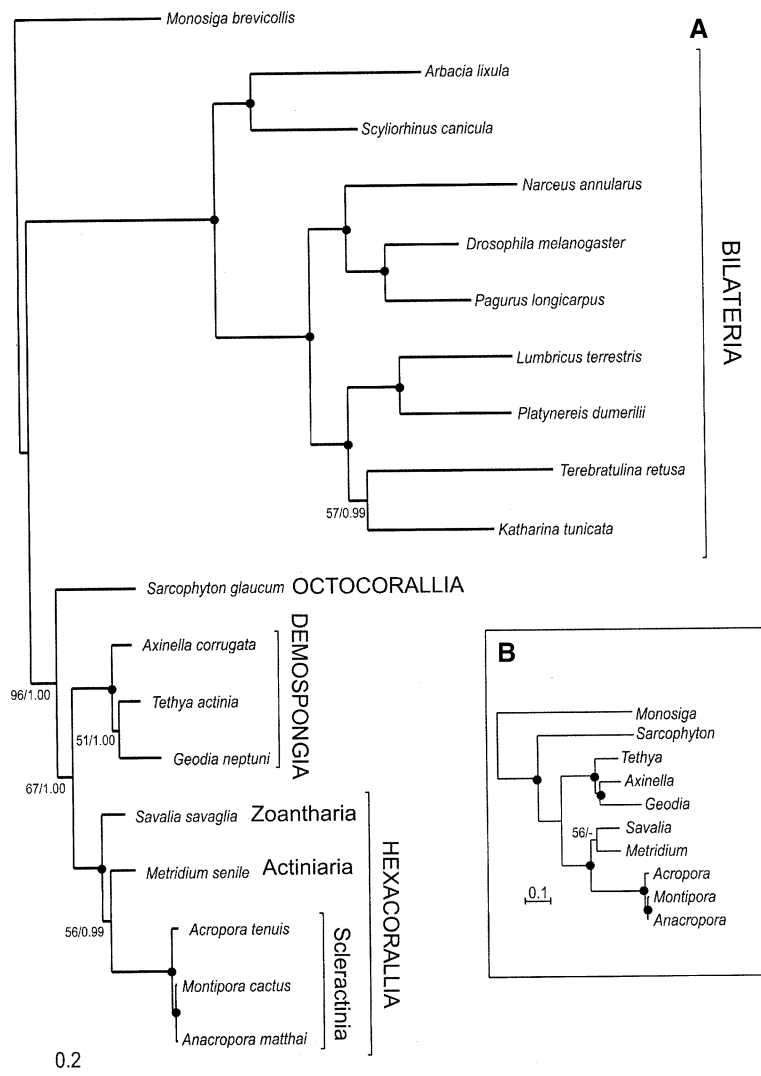
**Fig. 2.** Bayesian tree using Lavrov's alignment. Nodes fully supported by posterior probabilities are marked with a black circle. Weaker support values are indicated by the nodes.

## Conclusions

Our study shows the potential uses and limits of early metazoan mitochondrial genomes. On the one hand, the comparison of gene order in mitochondrial genomes provided important information on phylogenetic relationships among the three orders already sequenced (Scleractinia, Zooantharia, and Actiniaria). Although mitochondrial genomes from the other hexacorallian orders (Ceriantharia, Antipatharia, and Corallimorpharia) remain to be sequenced, there is good evidence that mitochondrial genomes may contribute significantly to the resolution of hexacorallian phylogeny. On the other hand, the mitochondrial genomes seem to have limited use for resolution at higher

taxonomic levels (phyla, classes) in early metazoans. However, increasing taxon sampling would be necessary to test if such characters as mitochondrial gene contents could not be used to explore the phylogeny at the dawn of animal evolution.

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**Fig. 3.** **A** Bayesian tree obtained with the second set of data, including 18 metazoan taxa for 2458 positions. The topology is identical to the ML tree. **B** Best ML tree obtained with diploblastic taxa only for 3500 sites. Nodes fully supported by bootstrap values and posterior probabilities are marked with a black circle. Weaker supports are indicated by values at the nodes.

**Table 1.** Separate analyses of each gene with maximum likelihood and status of Anthozoa

| Gene  | Number of sites | RRT <sup>1</sup> p-value <sup>a</sup> | Support Anthozoa monophyly <sup>b</sup> |
|-------|-----------------|---------------------------------------|---|
| ATP6  | 224             | 0.00636                               | No                                      |
| ATP8  | 41              | 0.00020                               | Yes                                     |
| COI   | 472             | 0.00000                               | No                                      |
| COII  | 221             | 0.00994                               | Yes                                     |
| COIII | 260             | 0.44920                               | No                                      |
| CytB  | 377             | 0.08243                               | Yes                                     |
| ND1   | 304             | 0.41775                               | No                                      |
| ND2   | 315             | 0.00000                               | No                                      |
| ND3   | 115             | 0.05525                               | Yes                                     |
| ND4   | 463             | 0.00858                               | No                                      |
| ND4L  | 99              | 0.00514                               | No                                      |
| ND5   | 539             | 0.00001                               | No                                      |
| ND6   | 133             | 0.02111                               | Yes <sup>c</sup>                        |
| 12S   | 705             | 0.00208                               | Yes                                     |
| 16S   | 1132            | 0.00000                               | Yes                                     |

<sup>a</sup> Relative rate test results considering *Savalia* and *Sarcophyton* as in groups and *Geodia* (Desmospongia) as outgroup.

<sup>b</sup> When the monophyly is not supported, *Sarcophyton* branches at the base of the diploblasts.

<sup>c</sup> However, *Sarcophyton* branches within Hexacorallia.

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