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Partial Sequence of a Sponge Mitochondrial Genome Reveals Sequence Similarity to Cnidaria in Cytochrome Oxidase Subunit II and the Large Ribosomal RNA Subunit

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Abstract. A 2550-bp portion of the mitochondrial genome of a Demosponge, genus *Tetilla*, was amplified from whole genomic DNA extract and sequenced. The sequence was found to code for the 3′ end of the 16S rRNA gene, cytochrome *c* oxidase subunit II, a lysine tRNA, ATPase subunit 8, and a 5′ portion of ATPase subunit 6. The Porifera cluster distinctly within the eumetazoan radiation, as a sister group to the Cnidaria. Also, the mitochondrial genetic code of this sponge is likely identical to that found in the Cnidaria. Both the full COII DNA and protein sequences and a portion of the 16S rRNA gene were found to possess a striking similarity to published Cnidarian mtDNA sequences, allying the Porifera more closely to the Cnidaria than to any other metazoan phylum. The gene arrangement, COII—$tRNA^{Lys}$—ATP8—ATP6, is observed in many Eumetazoan phyla and is apparently ancestral in the metazoa.

Key words: Porifera — Mitochondrial gene — Molecular systematics — Demospongiae — Phylogenetics — Metazoa — Diploblast — Genome evolution

Introduction

Phylum Porifera, the sponges, represents the most distantly diverged metazoan group. Diversification of the sponges almost certainly predates the Cambrian explosion (Gehling and Rigby 1996; Brasier et al. 1997; Li et al. 1998). As a basally derived metazoan lineage, synapomorphies with other metazoans will be particularly valuable in determining characteristics of the earliest multicellular animals. Sponges have already been shown to possess a wide range of genes in common with the rest of the metazoa, including multiple classes of homeobox genes (Seimiya et al. 1994), ETS transcription factors (Degnan et al. 1993), an endocrine signaling circuit (Robitzki et al. 1989), families of collagen genes (Exposito et al. 1991), receptor tyrosine kinases (Gamulin et al. 1997), and many others (Shenk and Steele 1993). All of these characters set the metazoa apart as a monophyletic lineage distinct from the protists, although some authors prefer to place the sponges into a distinct subkingdom, the Parazoa (Willmer 1990).

To date, only a single mitochondrial genome from the diploblast metazoa has been fully sequenced and published, that of the anthozoan *Metridium senile*, a hexacoral (Pont-Kingdon et al. 1994; Beagley et al. 1996). Unique features found in this genome include Group I introns, only two mitochondrially encoded tRNAs, and a prokaryote-like srRNA structure. The mitochondrial genome of another cnidarian, the octocoral *Sarcophyton glaucum*, has been shown to contain a unique ORF which codes for a gene homologous to a bacterial DNA mismatch-repair enzyme (Pont-Kingdon et al. 1995), which occurs as well in another octocoral, *Renilla kolikeri* (Beagley et al. 1995). Whether this has led to a lower rate of sequence evolution in the octocorals, and whether the presence of an active, partially mitochondrially encoded

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mismatch repair system is restricted to the Cnidaria, remains an open question (Wolstenholme 1992).

The existence of these unique features in the cnidarian mitochondrial genome sheds new light upon the evolution of metazoan mitochondrial DNAs. As metazoan mitochondrial genomes are considerably smaller and code for fewer genes than protist mitochondrial genomes, a reduction in genome size has obviously occurred. Since Cnidarian mitochondrial genomes appear to contain more and longer genes, it is possible that this size reduction occurred over a broad time scale. The characterization of mitochondrial genomes from other lower metazoan phyla (the Porifera and Ctenophora) would be valuable in elucidating the process whereby the present form of animal mitochondrial genomes was generated.

Similarly the genetic code of cnidarians has been found to lack all of the modifications from the universal code which are found in the triploblast metazoa, with the exception of the TGA = W modification (Wolstenholme 1992). As the Cnidaria are generally accepted to comprise a later radiation than the Porifera, the mitochondrial genetic code of the sponges is of interest in elucidating the evolution of metazoan mitochondrial genetic codes and in establishing the characteristics of the earliest metazoan state.

We report here the first characterization of a portion of the mitochondrial genome of a Demosponge, genus *Tetilla.* A 2550-bp portion of the mt genome was amplified and sequenced. The mitochondrial genetic code was found to be identical to that of the anthozoan *Metridium senile,* and analysis of the partial lrRNA gene and the full COII gene demonstrated an unusually high degree of similarity to cnidarian mtDNA as well. While the DNA and protein sequences of these genes are conserved relative to the *Metridium senile* sequence, their relative organization is not. The *Tetilla* sp. gene organization is closer to that of the octocoral *Renilla kolikeri* (Beagley et al. 1995).

Materials and Methods

Sponge Materials. Live sample organisms of *Tetilla* sp. (Class Demospongiae) and *Leucilla nuttingi* (Class Calcarea) were supplied by Seacology, Inc., of Vancouver, B.C. Samples of *Tetilla* sp. were cleaned of surface debris upon arrival and frozen at −80°C. Samples of *Leucilla nuttingi* were frozen at −80°C upon arrival. Samples of *Grantia* sp. (class Calcarea) preserved in 50% isopropanol were donated by Steve Halford (Simon Fraser University Natural History Collection). The taxonomies of the sponges used are detailed in Table 1.

The species status of the primary sponge sample is uncertain. It represents either *Tetilla spinosa* or *Tetilla villosa. Tetilla spinosa* may in fact represent an immature form of *Tetilla villosa* (B. Austen, personal communication), so the distinction may be irrelevant.

DNA Extraction. Whole cellular DNA extraction for all organisms was performed with the Isoquick DNA Extraction Kit (Microprobe Corporation). Total DNA was extracted from approximately 200 mg of tissue according to the manufacturer's protocol, using the total DNA and RNA extraction method.

Table 1. Classification of the sponges used in this study

Phylum Porifera
Class Calcarea
Subclass Calcaronea
Order Sycettida
Family Grantiidae
Grantia sp.
Family Amphoriscidae
Leucilla nuttingi
Class Demospongiae
Subclass Tetractinomorpha
Order Spirophorida
Family Tetillidae
Tetilla spinosa[a]
Tetilla villosa[a]

[a] Species identity uncertain; see Materials and Methods. Genus is also called *Craniella.*

PCR Amplification of mtDNA Bands. Amplification of candidate poriferan mtDNA bands was performed with reagents from the GeneAmp XL PCR Kit (Perkin–Elmer). Amplification of trans-gene regions of the mitochondrial genome was attempted with a variety of mitochondrial primers. A single 2.6-kb band was amplified from *Tetilla* sp. using the primer 16S1 (Bridge et al. 1992), which was designed to amplify the 5′ end of a 758-bp 16S rRNA band in *Metridium senile* (Cnidaria) and the primer CSC2R1, which was designed as a conserved cytochrome oxidase subunit II primer from a range of metazoan COII sequences including *Metridium senile* (Pont-Kingdon et al. 1994). The primer 16S2 (Bridge et al. 1992), also designed from cnidarian 16S rRNA sequence, allowed the amplification of an approximately 750-bp 16S band from whole genomic DNA of all of the species examined and from the purified 2.6-kb *Tetilla* sp. band. Sequences of all DNA primers are given in Table 2.

In order to obtain the 2.6-kb *Tetilla* sp. band, the PCR reaction was performed on a Precision Scientific GTC2 DNA Thermal Cycler using a program of 94°C for 30 s, 52°C for 30 s, and 72°C for 12 min. Thirty-five amplification cycles were performed, the final cycle being followed by a final 12-min incubation at 72°C. Amplification of the 2.6-kb band using standard PCR mixes and cycles was not successful, but worked consistently with the long PCR protocol.

Cloning. In order to clone the ends of the 2.5-kb band, the *Tetilla* sp. band was subjected to a *Sau*3AI digestion, blunt-ended, and cloned into the *Sma*I site of pUC18. Following this, the band was subjected to a partial *Sau*3AI digestion and cloned into the *Bam*HI site of pUC18. Random clones were sequenced until a complete overlapping set had been obtained. Multiple overlapping sequence reads from separate clones were obtained for almost every portion of the sequence.

Sequencing. Minipreps of the pUC18 clones were sequenced directly using the M13 Forward primer and USB's Sequenase Version 2.0 kit on 5% polyacrylamide gels in 0.5X TBE.

Once the full sequence of the 2.6-kb band had been obtained, further amplifications and sequencing reactions were performed using primers developed from this sequence and matching primers extant in the laboratory. These primers are listed in Table 2. Sequencing of bands amplified from genomic DNA using these primers was performed using Amersham's Thermo-Sequenase cycle sequencing kit on a Perkin–Elmer/Cetus GeneAmp PCR System 2400 machine using the kit manufacturer's protocols.

Results and Discussion

Gross Characterization of the Sequence. The full sequence of the 2550-bp demosponge mtDNA band (Fig.

Table 2. Sequences of DNA primers used in this study

Primer	Sequence
CS16S3	5′-GTATGAATGGCTTAACGA[a]
N	5′-ATCCAACATCGAGGTCG[b]
16S4	5′-TAGGGATAACAGCGCAAT[c]
16S1	5′-TCGACTGTTTACCAAAAACATAGC[d]
16S2	5′-ACGGAATGAACTCAAATCATGTAAG[d]
CSC2R2	5′-TTAAAGCAAACTTTTCCA[a]
CSC2R1	5′-TGATTAGCCCCRCARAT[a]
V	5′-CGTTCCGGTTGATAACCTCATC[b]
CSC2F3	5′-AATGTTCATATTAACTAT[a]
CSC2F1	5′-TGGAATTTGAYTCTTAYAT[a]
CSC2F2	5′-CATTCTTTTATGCCWATTGT[a]
CSA8F1	5′-GTCAGTATATATGAAAGT[a]
CSA6F1	5′-AACAGATGGCAATTTCTT[a]
CSA8R1	5′-TTAACATGGCAATAAAGA[a]

[a] Developed from the *Tetilla* sp. sequence.
[b] Liu (1993).
[c] Wei (1992).
[d] Bridge et al. (1992).

1) reveals the presence of three open reading frames at positions 997–1737, 1825–2015, and 2016–2550. Analysis of the first ORF reveals a very high sequence similarity, at both the nucleotide and the predicted amino acid sequence level, to the previously published sequences of mitochondrial cytochrome oxidase subunit II from *Metridium senile* (Pont-Kingdon et al. 1994) and from a wide range of other metazoan and nonmetazoan taxa. The third ORF, at positions 2016–2550, reveals sequence similarity to mitochondrial ATPase subunit 6 from a variety of metazoan and nonmetazoan taxa. The second ORF cannot be easily identified on the basis of DNA sequence. The DNA sequence from base 1 to approximately base 844 exhibits a high degree of similarity to the 3′ end of the mitochondrial large ribosomal RNA subunit in a wide variety of taxa, and the sequence from bases 1751 to 1823 demonstrates similarity to *E. coli* tRNA-Lys and a variety of other prokaryotic tRNAs.

Verification of Amplified DNA Source. Our immediate goal was to assess whether the sequence represented true poriferan mitochondrial DNA or DNA amplified from a symbiotic or contaminating organism. Many sponges are known to harbor dense intracellular populations of zooxanthellae, zoochlorellae, and cyanobacteria (Simpson 1984). Additionally, since the DNA extraction was made from whole tissue, the coextraction of DNA from small marine organisms inhabiting the internal canals and choanocyte chambers in the sponge or from larvae of marine organisms including echinoderms, amphipods, annelids, and copepods was a significant possibility.

The full amino acid sequences of the COII genes from five metazoan phyla along with one fungal COII sequence and the COII sequences of a red algae and a green algae were aligned. The inferred amino acid sequence of the candidate poriferan COII band was aligned with these sequences as well, using the cnidarian mt genetic code for translation (Pont-Kingdon et al. 1994), as it appeared to provide a translation consistent with the positions of conserved amino acids in the other COII genes (see Discussion below). Alignments were performed first with CLUSTALV (Higgins 1994) and then inspected by eye to optimize the alignment. Alignments of the DNA sequences of these genes were then made using the amino acid alignments.

Results of neighbor-joining analysis of both the amino acid and the DNA alignments are shown in Fig. 2. The phylogenies recovered from both data sets are consistent with one another and show a very high bootstrap support (>95% in all cases) for placement of the candidate poriferan sequence as a sister taxon to the one published cnidarian COII sequence, that of the anthozoan *Metridium senile.* This placement is supported as well by maximum-parsimony analyses on both the nucleotide and the amino acid data, although at only 77 and 65% of bootstrap replicates with DNA and amino acid parsimony respectively. In both cases, however, the sequence is supported as metazoan at >98% of bootstrap replicates. Metazoan origin is also supported by DNA maximum-likelihood analysis.

In order to ensure that the mitochondrial sequence was repeatably obtained from this species in general, rather than from the one individual, the sequence from base 1654 to base 2286 was amplified from three other sponge individuals. This region includes the 3′ end of the COII gene, a putative tRNA gene, and the 5′ end of the putative ATPase subunit 8 gene. In all cases a band with identical sequence was produced, although there was some evidence of heteroplasmy at the 3′ end of COII in two individuals.

Since both of the primers utilized in amplifying this sequence were developed partially from cnidarian mitochondrial sequence data, the results of this analysis leave some concern that the product could represent an amplified contaminating cnidarian mitochondrial sequence. In order to address this issue, a portion of the mitochondrial large ribosomal RNA subunit gene was taken from the amplified candidate sequence and, also, amplified from a fresh-water calcisponge (*Grantia* sp.) and a marine calcisponge (*Leucilla nuttingi*). We reasoned that it would be unlikely that a common cnidarian contaminating species would be present in all three of these animals and, further, that comparison of this sequence with the same region from representatives of most cnidarian classes should place sponge sequence basally to the cnidarian radiation, provided that the sequence did in fact represent true poriferan mitochondrial DNA.

Partial mitochondrial large ribosomal RNA subunit sequences were retrieved from Genbank (Table 3). These sequences were aligned with partial mitochondrial lrRNA sequences obtained from *Tetilla* sp., *Grantia* sp., and *Leucilla nuttingi* (Table 3) with the 16S1 and 16S2

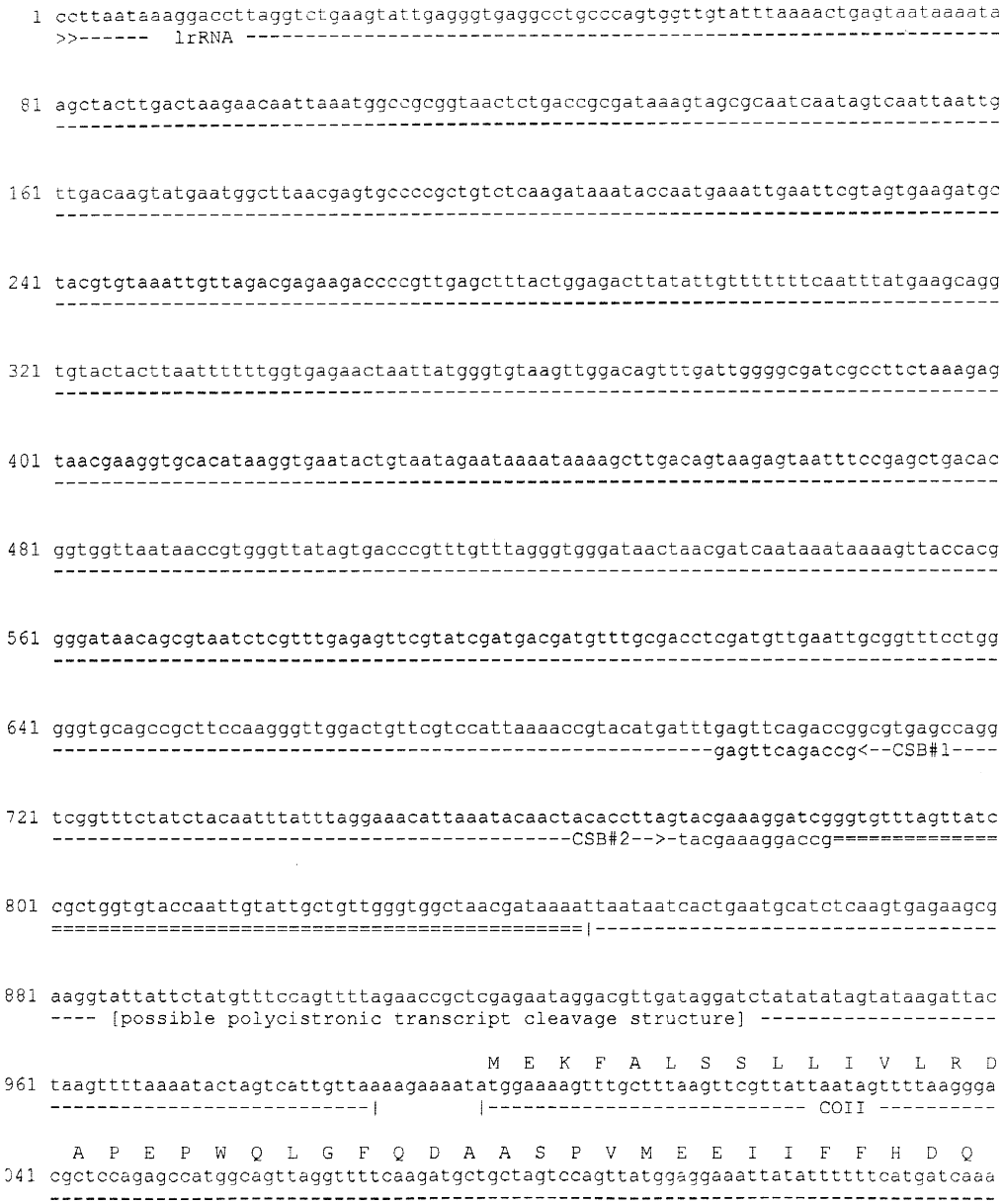

Fig. 1. Nucleotide sequence of the 2550-bp *Tetilla* sp. (Demospongiae) band. The sequence contains three protein-coding genes: cytochrome *c* oxidase subunit II (COII), ATPase subunit 8 (ATP-8), and the 5′ portion of ATPase subunit 6 (ATP-6). In addition, it contains the 3′ end of the large ribosomal subunit RNA gene (16S rRNA) and a single tRNA with a TTT anticodon for lysine. The sequence has been submitted to GenBank with the accession number AF035265. The exact 3′ end of the lrRNA gene is uncertain, but likely downstream of the two conserved sequence blocks CSB# 1 and CSB# 2 and upstream of the candidate polycistronic transcript cleavage site. This region is indicated with a string of = symbols. The TTT anticodon of the tRNA gene is indicated with three asterisks. The sequence appears to be the sole sense strand for all genes in this sequence, as all genes are encoded 5′ to 3′ in the sequence shown. Presumptive amino acid translations for all protein-coding genes are shown. The primer sequence is not shown.

primers, using CLUSTALV with a fixed gap weight penalty of 25 and a floating gap penalty of 25. Regions of unambiguous sequence alignment were selected for analysis, corresponding approximately to the three high-identity sequence blocks of France (1996).

Alignments were analyzed by neighbor joining using MEGA (Kumar et al. 1993) with both complete deletion of gaps and pairwise deletion, using the Kimura two-parameter correction. Five hundred bootstrap replications were performed, and the sponge sequences were found to represent a radiation basal to the Anthozoa, likely the most basally derived cnidarian class (Bridge et al. 1995) at 99% of bootstrap replications (Fig. 3). Bootstrap values for branchings within the Cnidaria are generally very low, due largely to the fairly small length of the sequence analyzed, and the position of the echinoderm sequence is erroneous due to its higher rate of sequence change. Inclusion of the more variable regions

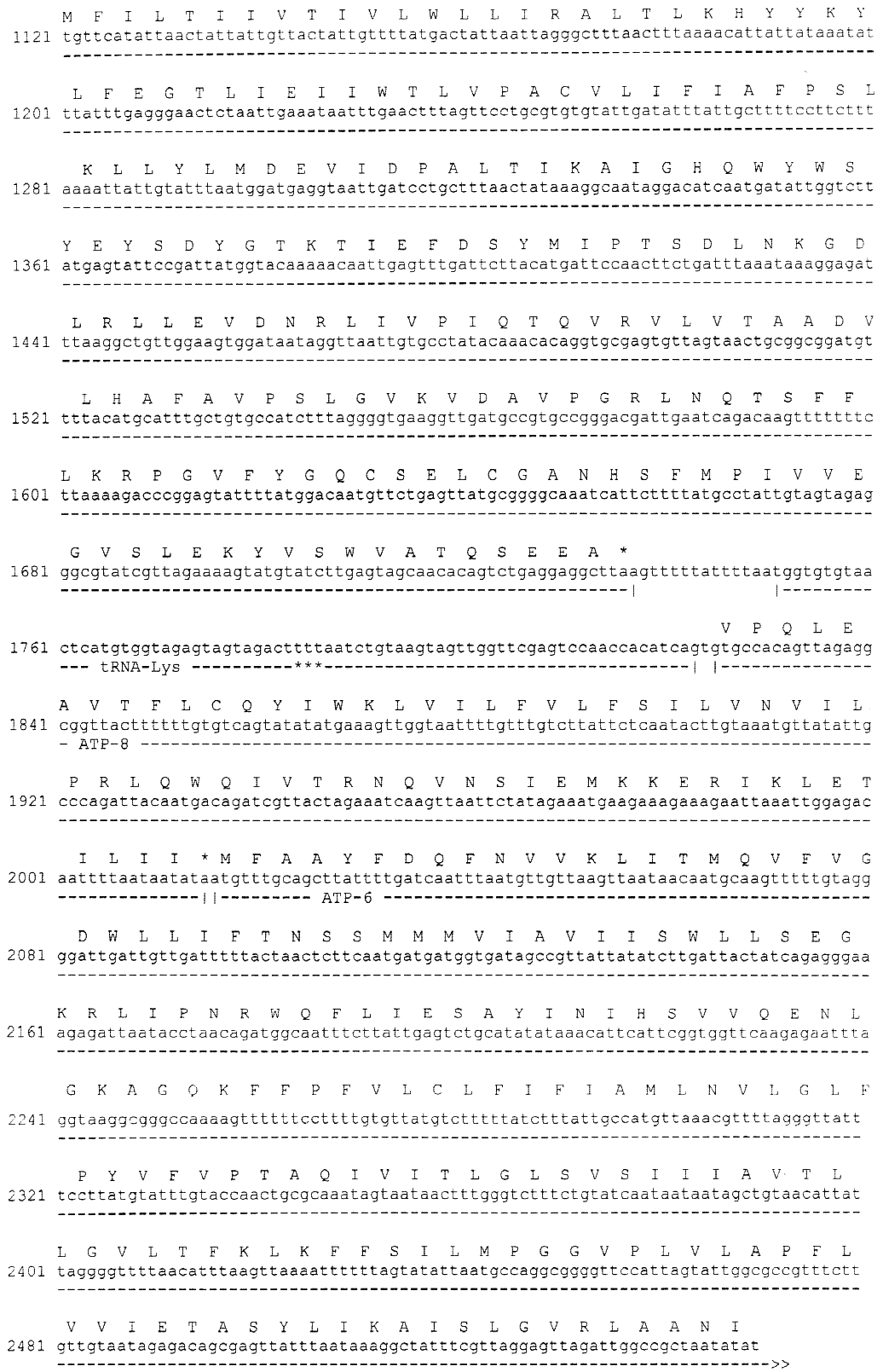

Fig. 1. Continued.

of the lrRNA sequences in this analysis further lowers the bootstrap values within the Cnidaria but maintains the relative position of the Porifera, although the branch length leading to the poriferan sequences increases significantly. For all analyses, essentially identical trees were obtained with other distance correction methods including gamma distances.

The full lrRNA data set, including the more highly

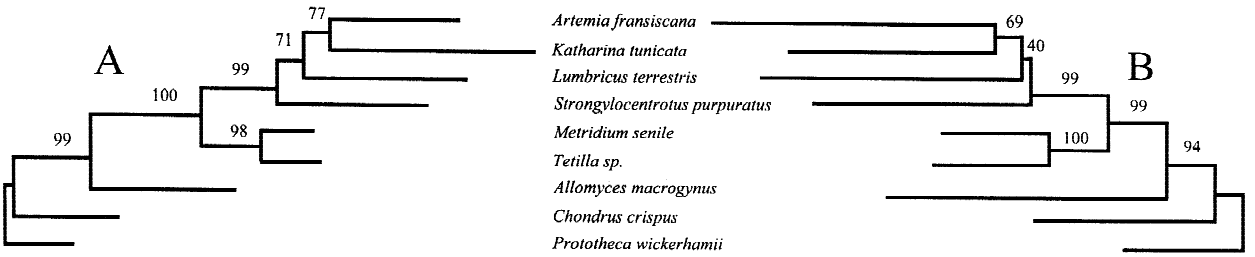

Fig. 2. Neighbor-joining trees of aligned COII DNA and amino acid sequences. Number show percentages of 500 bootstrap replications which support that branch. **A** Neighbor-joining tree derived from DNA sequence alignments using codon positions 1 and 2 only, and the Kimura two-parameter correction using only transversions and complete deletion of sites with gaps. **B** Neighbor-joining tree derived from amino acid sequence data using Poisson distances and complete deletion of sites with gaps. Accession numbers for sequences are listed in Table 3.

Table 3. Accession numbers of sequences used in this study

Source species	Gene	Classification	Accession No.
Anthopleura elegantissima	16S	Cnidaria: Anthozoa	U40292
Renilla muelleri	16S	Cnidaria: Anthozoa	U19372
Leptogorgia virgulata	16S	Cnidaria: Anthozoa	U19371
Antipatharia sp.	16S	Cnidaria: Anthozoa	U40287
Stichopathes spiessi	16S	Cnidaria: Anthozoa	U40286
Craterolophus convolvulus	16S	Cnidaria: Scyphozoa	U19375
Haliclystus sp.	16S	Cnidaria: Scyphozoa	U19376
Aurelia aurita	16S	Cnidaria: Scyphozoa	U19373
Casseiopia sp.	16S	Cnidaria: Scyphozoa	U19374
Liriope tetraphylla	16S	Cnidaria: Hydrozoa	U19377
Obelia dichotoma	16S	Cnidaria: Hydrozoa	U19378
Tubularia indivisa	16S	Cnidaria: Hydrozoa	U19379
Strongylocentrotus purpuratus	16S	Echinodermata: Echinoida	X12631
Tetilla sp.	16S	Porifera: Demospongiae	AF035265
Grantia sp.	16S	Porifera: Calcarea	AF035267
Leucilla nuttingi	16S	Porifera: Calcarea	AF035266
Prototheca wickerhamii	COII	Chlorophyta: Chlorophyceae	U02970
Chondrus crispus	COII	Rhodophyta: Florideophyceae	Z47547
Allomyces macrogynus	COII	Fungi: Chytridiomycota	U41288
Tetilla sp.	COII	Porifera: Demospongiae	AF035265
Metridium senile	COII	Cnidaria: Anthozoa	S75445
Strongylocentrotus purpuratus	COII, A8, A6	Echinodermata: Echinoida	X12631
Lumbricus terrestris	COII, A8, A6	Annelida: Clitellata	U24570
Katharina tunicata	COII	Mollusca: Polyplacophora	U09810
Artemia fransiscana	COII	Arthropoda: Crustacea	X69067
Drosophila melanogaster	COII, A8, A6	Arthropoda: Insecta	U37541
Homo sapiens	COII, A8, A6	Chordata: Vertebrata	J01415

variable regions, was analyzed by maximum parsimony using PAUP 3.1.1 (Swofford 1993). Using the heuristic search option, two most-parsimonious trees were found, both of which grouped the Porifera separately from the Cnidaria. In neither case was the placement of the poriferans as a separate radiation from the cnidarians affected, and in both cases the cnidarians, echinoderms, and poriferans represented an unresolved polytomy on the tree. A bootstrap 50% majority-rule consensus tree based upon 100 replications placed the Porifera as a separate radiation to the Cnidaria at 100% of replications (Fig. 4).

In order to investigate the effect of alignment upon these results a series of alignments was generated with CLUSTALV by varying the fixed and floating gap penalties (10/10, 10/25, 25/10, 25/25, 50/10, and 50/25). These complete alignments were analyzed by neighbor-joining with MEGA using the Kimura two-parameter correction and complete deletion of sites with gaps. In all cases the Porifera were found to cluster as a separate radiation from the Cnidaria, although the exact placement of the cnidarian classes with respect to one another varied somewhat, as did the placement of the echinoderm sequence.

Protein-Coding Genes. The ORF from bases 997 to 1737 codes for cytochrome *c* oxidase subunit II. Comparison with other mitochondrial COII genes from both metazoan and nonmetazoan taxa (Fig. 5) reveals that the

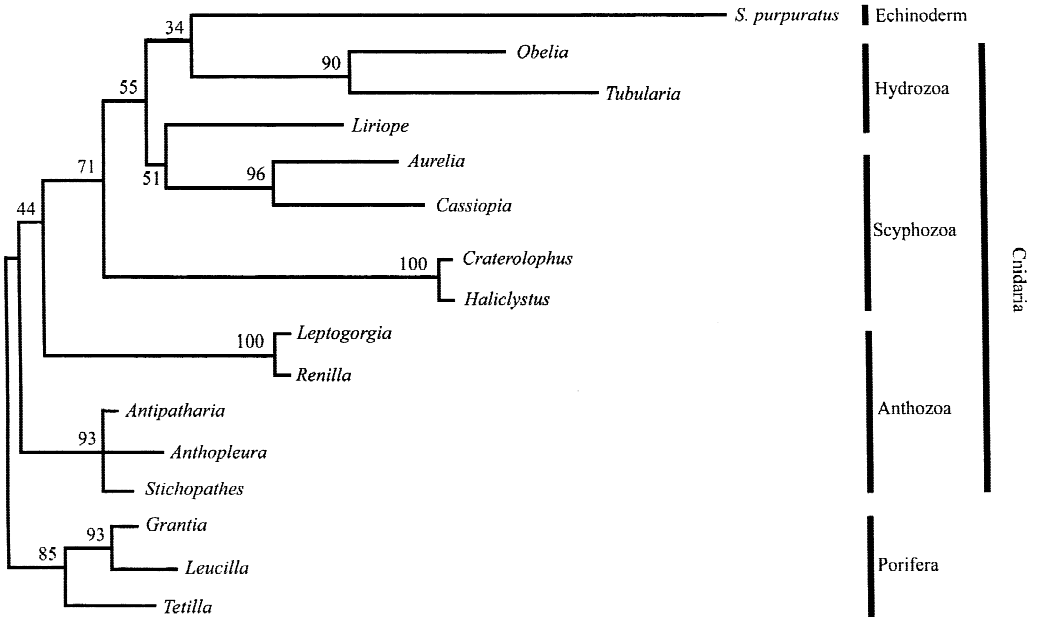

Fig. 3. Neighbor-joining tree derived from short (~750-bp) Cnidarian, Echinoderm, and candidate Poriferan mitochondrial 16S rRNA sequences using the Kimura two-parameter correction, transversions only, and complete deletion of sites with gaps. Use of other distance correction parameters was found to have no significant effect upon either branch order or branch length. Only the three highest-confidence blocks of conserved sequence were utilized to produce this tree. *Numbers* show percentages of 500 bootstraps which show support for that branch. The placement of the Echinoderm sequence is aberrant and is likely a result of a combination of a much higher relative rate of sequence evolution and lower confidence sequence alignment.

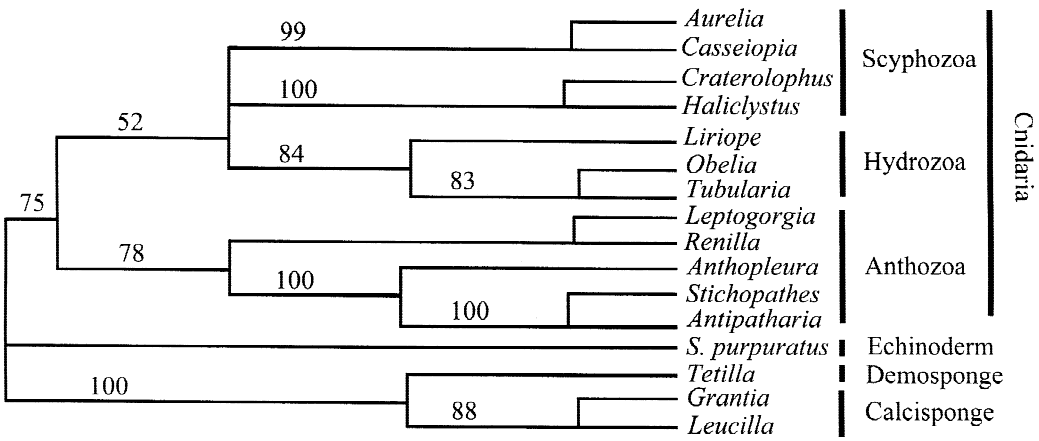

Fig. 4. Bootstrap 50% majority-rule consensus tree derived from Cnidarian, Echinoderm, and candidate Poriferan mitochondrial 16S rRNA sequences by maximum parsimony. The full (~750-bp) sequence alignments were utilized to produce this tree. *Numbers* show the percentage support for that branch of 100 bootstrap replications.

poriferan sequence shares a common 5′ insertion with Cnidaria relative to the rest of the metazoa, although the sponge insertion is two amino acids shorter than the cnidarian sequence. The presence of this 5′ sequence appears to be a shared feature with both the fungal and the algal COII genes, suggesting that a deletion of some 17 amino acids occurred following the divergence of the triploblast lineage away from the diploblasts. The metazoa are distinguished from the fungal and algal sequences, however, by the common presence of a deletion at base 1201 of the COII sequence, corresponding to a six-amino acid deletion relative to the fungi and a five-amino acid deletion relative to the algae. Both diploblast genes are longer than the triploblast mitochondrial COII gene (Table 4).

The ORF at bases 2016 to 2550 shows a high degree of similarity to the 5′ 178 amino acids of ATPase Subunit 6 (Fig. 6) from a wide range of taxa, but it is not possible to estimate the full length of the gene and its size relative to other metazoan taxa with this partial sequence. The ORF at bases 1825–2015 may code for ATPase subunit 8, based solely upon the hydropathy profile of its inferred amino acid sequence (Fig. 6). If this is the case, then the gene appears to overlap the 5′ end of the ATPase

```
Tetilla       MEKFALSSLLIVLR---DAPEPW-QLGFQDAASPVMEEIIFFH-DQIMFILTIIVTIVLWLLIRALTLKHYYKY------ 69
Metridium     .TNL-.NNW..INQFGY.L....-...L....H..........-..V....I..I.T....IVK..SG.A.HR.------ 71
Strongyloc    .GT-------------------.A.F.L...S..L...LTY..-.YALIV..L.TIL.FYG.VSL.VSSNTNRF------ 54
Artemia       VSQ-------------------.F...L.NGN..L..QL....-.HALLVVIL.TSL.GFF.AALFSN.FLHR.------ 54
Katharina     .AF-------------------.S.W....G...I..QL....-.HA.L..IM.ISLLSYGAVSLMNNSFLSRS------ 54
Drosophila    .ST-------------------.AN..L..S...L..QL....-.HALL..VM.TVL.GY.MFMLFFNNYVNRF------ 54
Lumbricus     .PN-------------------.G.VM......S..LQLVS..-.HALLV..LVL.V.GYA.LALMLN.QVNR.------ 54
Allomyces     .KLLNFIY--------N.H.Q..-.M....G.TTTFYG.VDL.-.N.I.Y.I.VIIG.T.IQTSVM.DSYGNSDKHVYKY 70
Chondrus      .NNILNFY---PAVITT.VA.N.-.I....P.T.I..G..NL.Y.-L..FICV.SVF.S.M.G.T.WHFEQNQNKIPSS- 74
Prototheca    .KFLLFAYA..PFVSFS....A.-.I....P.T.I.QGL.DL.H.-.Q.F.IAVLVF.V.MVS...Y.F..TRNPLPEK- 77

Tetilla       LFEGTLIEIIWTLVPACVLIFIAFPSLKLLYLMDEVIDPALTIKAIGHQWYWSYEYSDY---GTKTIEFDSYMIPTSDLN 146
Metridium     .VD...L.....I...II..L..............M.....................---Q.E.L......V...E.. 148
Strongyloc    F...QEL.T...VI..LI..L..L...Q........N..F.....F..........T.FN-----DL......V....VS 129
Artemia       .LD.QA..TV..VI..II.VA..L..IR....I..IHN......VT...........LN-----D.Q.......SNE.S 129
Katharina     TL.SQE...V..IL..V....L.....Q....L..LEE......VV...........FL-----NL.......SLE..E 129
Drosophila    .LH.Q...M...IL..II.L...L...R....L..INE.SV.L.S...........FN-----N..........NE.M 129
Lumbricus     IM.AQTV.T...IL..LI.LVL.L...RI..IT...SQ.SI.V.T..........T.FL-----NV.M....L.....L 129
Allomyces     SHH...V.....IT..LI.VA.....F....T..S.V...V.V....Y....G.N.G..NE----D.S...F.V..DE.E 146
Chondrus      .TH.....M...VT..FI.LI...V..FS...A...I.S..I...TL............LNEDDDS.NY......EE..E 154
Prototheca    IIH......V..IT.SLI.....V..FA...SL...V...V..................SIADDQS.A.......DD..E 157

Tetilla       KGDLRLLEVDNRLIVPIQTQVRVLVTAADVLHAFAVPSLGVKVDAVPGRLNQTSFFLKRPGVFYGQCSELCGANHSFMPI 226
Metridium     ...F.........V...N.H......G.....S....A.A..M..I.......G..I....I........I.......... 228
Strongyloc    F.NP.........VL.M.NPI....SS.....SW......T.M..........T..AA.T.........I.......... 209
Artemia       T.MY...D....SQC.MIKAI.LMI.SDA...SW......I.M..D......S.LLVNM..........I..SG...... 209
Katharina     E..Y......H.SV..MK.K............SWT.......A.........L...ANY..........I.......... 209
Drosophila    TDGF...D....VVL.MNS.I.I.......I.SWT..A......GT.......N..IN...L.......I.......... 209
Lumbricus     P..Y........MV..M.LEI.M.I.....I.SWT..A..............IG.TTTQ..........I.......... 209
Allomyces     M.QF........VV..VD.R..IV......I.S.......I.I..I...I..A..LID.E..Y......T.....G.... 226
Chondrus      I.QF........MVI..N.HI..I........SW....FRS.C..I........L.I..E..Y......I..I..G.... 234
Prototheca    L.QY........VV..VD.HI..II.......SW.I......C.........IPM.I..E...........T..A.... 237

Tetilla       VVEGVSLEKYVSWVATQSEEA                                                          247
Metridium     .I.A...D..IN..I.G.D.-                                                          248
Strongyloc    .I.S.PFNTFEN..TQYL..-                                                          229
Artemia       .I.A.GESDFLK.LEL.I-S-                                                          228
Katharina     .L.V.DSSSFIK.IMFNG.A-                                                          229
Drosophila    .I.S.PVNYFIK.ISSN-NS-                                                          228
Lumbricus     A..AINTKSFM...S-NFKP-                                                          228
Allomyces     AI.A..TDR..A..DA.L-S-                                                          245
Chondrus      ...AT..PN....ISNKLN.-                                                          254
Prototheca    ...A....N.I...SNKL..L                                                          258
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Fig. 5. Alignment of the full demosponge COII gene with representative COII genes from both metazoan and nonmetazoan taxa. Note both the 5′ indel in common between the triploblast metazoan phyla and the smaller indel, which sets the metazoan sequences as a whole apart from the nonmetazoan sequences. Amino acid residues identical to the *Tetilla* sp. sequence are indicated by a *dot,* and gaps relative to the *Tetilla* sp. sequence are indicated by a *dash.* Accession numbers for sequences are listed in Table 3.

subunit 6 gene by one nucleotide. This situation is known from many other metazoan phyla (Wolstenholme 1992).

Codon Usage, Nucleotide Bias, and Genetic Code. The frequencies of nucleotides in the third codon position of the three protein-coding genes is proportional to their relative usage in the full mtDNA segment (Tables 5 and 6). For third codon positions, codons ending in A or T constitute 74.6% of those used, compared to 59.3% of first codon positions and 68.7% of second codon positions. This suggests an overall stronger AT bias in third codon positions relative to AT content of the sequence as a whole (66.2%). Of the nine codons not found to be used in this segment of the mitochondrial genome, eight of them end in either G or C, the exception being CGT (arginine).

All of the codons whose specificity most frequently changes are found in the COII gene, and comparison of the COII nucleotide and amino acid sequences from *Tetilla* sp. with a variety of other taxa allows a reasonable estimate of their translations to be made. In the case of *Metridium senile* (Pont-Kingdon et al. 1994) evidence regarding the assignment of the TGA codon to tryptophan rather than a stop codon was found to be inconclusive, but the authors felt that their translations were consistent with TGA being assigned to tryptophan. Further sequence analysis has supported this interpretation (CT Beagley, personal communication). In the case of *Tetilla* sp., a TGA codon occurs four times, and in three of those cases the tryptophan residue at that position is universally conserved in the COII sequences of taxa ranging from *Drosophila melanogaster* to *Prototheca wickerhamii.* In each of these cases the *Metridium senile* COII sequence contains a TGG codon for tryptophan at that position. The TGA codon then is likely to be translated as tryptophan in *Tetilla* sp.

The mitochondrial genetic codes of higher metazoan phyla commonly utilize methionine as the translation for ATA codons, but the translation of this codon in turbellarian, platyhelminths, and echinoderms is isoleucine,

Table 4. Comparison of *Tetilla* sp. mtDNA complete protein-coding genes with those of other taxa

	ATP 8 length (amino acids)	COII length (amino acids)	% COII amino acid identity			
			With *Tetilla* sp.	With *M. senile*	With *D. melanogaster*	With *P. wickerhamii*
Tetilla sp.	63	247	—	74.4	53.0	59.3
Metridium senile[a]	72[k]	248	74.4	—	55.2	55.4
S. purpuratus[b]	55	229	57.1	57.3	61.6	46.3
C. elegans[c]	NF[l]	231	37.1	38.6	43.2	39.8
D. melanogaster[d]	53	228	53.0	55.2	—	47.1
K. tunicata[e]	53	229	56.3	53.6	62.4	48.8
Lumbricus terrestris[f]	53	228	50.6	49.2	59.6	46.3
Artemia franciscana[g]	53	228	47.4	49.2	61.8	43.6
Allomyces macrogynus[h]	47	245	54.5	55.7	45.5	54.8
Chondrus crispus[i]	NF	254	54.9	53.9	47.8	64.7
P. wickerhamii[j]	NF	258	59.3	55.4	47.1	—

[a] Pont-Kingdon et al. (1994).
[b] Jacobs et al. (1988).
[c] Okimoto et al. (1992).
[d] de Bruijn (1983).
[e] Boore and Brown (1994).
[f] Boore and Brown (1995).
[g] Perez et al. (1994).
[h] Paquin and Lang (1996).
[i] Leblanc et al. (1995).
[j] Wolff et al. (1994).
[k] CT Beagley, personal communication.
[l] NF, not found.

that of the universal genetic code. In *Metridium senile* evidence was found that the translation of ATA codons was also for isoleucine (Pont-Kingdon et al. 1994). In *Tetilla* sp., there are 8 ATA codons; in the COII nucleotide sequences of *Strongylocentrotus purpuratus, Katharina tunicata, Artemia franciscana, Drosophila melanogaster,* and *Lumbricus terrestris,* there is a total of 35 codons corresponding to 7 of these positions. Isoleucine is found at 11 of these positions in the amino acid sequence compared to methionine, which is found only six times. Two of these positions are well conserved for isoleucine and show no methionine codons in any of the other sequences, even when fungal (*Allomyces macrogynus*), red algal (*Chondrus crispus*), and green algal (*Prototheca wickerhamii*) COII sequences are included in the analysis. It seems reasonable to infer that the mitochondrial ATA codon in *Tetilla* sp. codes for isoleucine, the universal genetic code translation.

The codon AAA has been found to maintain its standard genetic code assignment of lysine in all metazoan phyla with the exception of the echinoderms and platyhelminths, in which it codes for asparagine. Cnidarians appear to maintain the standard genetic code assignment (Wolstenholme 1992). In the case of the sponge COII gene, four AAA codons are found, one of which corresponds to the position of an AAG-encoded lysine residue in *Metridium senile,* and AAA-encoded lysine residues in *Artemia* and *Lumbricus,* and two of which correspond to presumptive AAA-encoded lysine residues in *Metridium senile.* Although there are no strongly conserved lysines at any of these positions, the results are consistent with AAA codons specifying lysine in *Tetilla* sp.

The two codons AGA and AGG are the most frequently reassigned in the mitochondrial genetic code of metazoans. While in the universal code they specify arginine, they are variously reassigned throughout the higher metazoan lineages. The *Metridium senile* mitochondrial genetic code was found (Pont-Kingdon et al. 1994) to utilize the standard genetic code specificity of arginine. In the case of the *Tetilla* sp. mitochondrial COII gene, there is only a single AGA codon, which corresponds to an AGA codon in *Metridium senile.* This codon position codes for an arginine residue in *Strongylocentrotus purpuratus* and in the fungal and algal lineages as well. This being the only AGA codon in this gene, and the position not being very well conserved in the metazoa, the only conclusion which can be drawn is that its usage is consistent with AGA specifying arginine. Three examples of the AGG codon are found in *Tetilla* sp., two of which correspond to very highly conserved arginine residues, with perfect conservation in all of the species utilized in the alignments. These two positions are coded for in *Metridium senile* by AGA codons.

Three initiation codons are found in this mitochondrial sequence: for COII, ATP8, and ATP6. In two cases an ATG initiation codon is found, whereas the ATP8 gene appears to begin with a GTG initiation codon. The use of GTG instead of ATN as a mitochondrial initiation

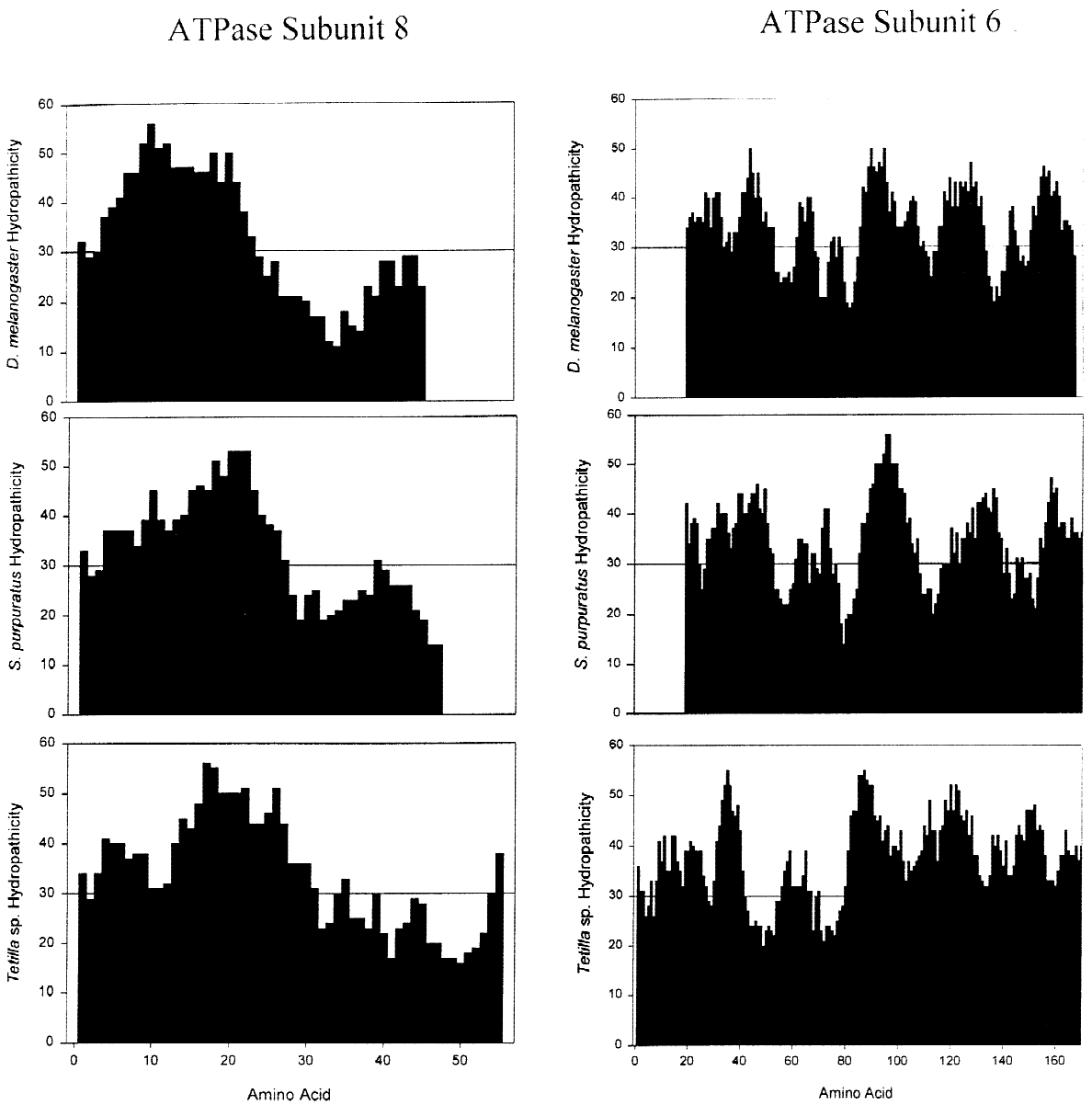

Fig. 6. Kyte–Doolittle hydropathy plot of presumptive ATP-8 and ATP-6 sequences from *Tetilla* sp. compared with hydropathies of the same genes from *Drosophila melanogaster* and *Strongylocentrotus purpuratus.* All hydropathies were calculated using the GREASE program from the FASTA package (Pearson and Lipman 1988).

Table 5. Nucleotide composition of *Tetilla* sp. COII, ATP8, and ATP6 genes compared with that of other metazoans

Species	% T	% A	% G	% C
Tetilla sp.[a]	37.83 (34.67)	29.65 (30.07)	20.31 (21.61)	12.21 (13.65)
M. senile[b]	34.94	29.32	19.54	16.20
S. purpuratus	32.24	27.00	16.60	24.16
L. terrestris	31.18	30.07	13.88	24.87
D. melanogaster	42.34	33.69	11.05	12.92
H. sapiens	24.79	30.78	11.78	32.65

[a] Numbers in parentheses refer to the percentage nucleotide composition of mtDNA band as a whole.
[b] COII gene only for *Metridium senile.*

codon is infrequent in metazoa but is known to occur in chordates and echinoderms, but not in arthropods, nematodes, or cnidarians (Wolstenholme 1992).

The mitochondrial termination codons of most metazoan phyla have been found to include TA or T partial termination codons to which 3′ adenines are added by polyadenylation of the RNA transcript (Ojala et al. 1981). The mitochondrial genome of *Metridium senile*

Table 6. Absolute and relative frequency of codons in the protein-coding portions of the mitochondrial fragment[a]

	Num.	Rel. %		Num.	Rel. %		Num.	Rel. %		Num.	Rel. %
Phe			Ser			Tyr			Cys		
TTT	32	6.5	TCT	14	2.8	TAT	15	3.0	TGT	4	0.8
TTC	2	0.4	TCC	1	0.2	TAC	1	0.2	TGC	1	0.2
Leu			TCA	4	0.8	TER			Trp		
TTA	44	8.9	TCG	4	0.8	TAA	2	0.4	TGA	8	1.6
TTG	14	2.8				TAG	0	0.0	TGG	3	0.6
Leu			Pro			His			Arg		
CTT	6	1.2	CCT	8	1.6	CAT	6	1.2	CGT	0	0.0
CTC	0	0.0	CCC	2	0.4	CAC	0	0.0	CGC	0	0.0
CTA	3	0.6	CCA	9	1.8	Gln			CGA	2	0.4
CTG	1	0.2	CCG	2	0.4	CAA	13	2.6	CGG	0	0.0
						CAG	7	1.4			
Ile			Thr			Asn			Ser		
ATT	22	4.4	ACT	13	2.6	AAT	10	2.0	AGT	5	1.0
ATC	2	0.4	ACC	0	0.0	AAC	4	0.8	AGC	0	0.0
ATA	29	5.9	ACA	10	2.0	Lys			Arg		
Met			ACG	0	0.0	AAA	9	1.8	AGA	7	1.4
ATG	14	2.8				AAG	12	2.4	AGG	4	0.8
Val			Ala			Asp			Gly		
GTT	21	4.2	GCT	13	2.6	GAT	13	2.6	GGT	4	0.8
GTC	1	0.2	GCC	4	0.8	GAC	1	0.2	GGC	3	0.6
GTA	19	3.8	GCA	6	1.2	Glu			GGA	8	1.6
GTG	11	2.2	GCG	8	1.6	GAA	7	1.4	GGG	6	1.2
						GAG	16	3.2			

[a] Num, number of times the codon occurs in the 1467 bases of the protein-coding sequence in the 2550-bp sponge mitochondrial band. Rel %, relative percentage frequency of that codon in the total sequence. The only change from the universal genetic code appears to be the use of TGA to specify tryptophan, rather than as a termination codon.

showed no such partial stop codons (Wolstenholme 1992). The mitochondrial sequence characterized here for *Tetilla* sp. includes only the 3′ ends of two protein-coding genes (COII and ATP8), and both appear to end in a complete TAA termination codon (Fig. 1). The TAA terminator of ATP8 overlaps with the putative start (ATG) of ATP6 and would require alternate processing of a polycistronic transcript to translate both genes.

The lrRNA Gene. The sequence from bases 1 to 844 is similar to the 3′ end of the mitochondrial lrRNA gene from a wide range of metazoan taxa. It is not possible with the sequence data alone to determine the precise location of the 3′ end of the gene or make any inferences regarding its length relative to other metazoans. Two small conserved sequence blocks (Fig. 1) exist which are present at the 3′ ends of lrRNAs in a variety of taxa ranging from ciliate protists to *Drosophila melanagaster*. This suggests that the 3′ end of the lrRNA gene could exist nearer to these blocks than bordering the 5′ end of the COII gene. Also, there is a 143-base sequence from base 846 to base 988 which can be folded into a three-armed structure similar to sequences which may direct cleavage of polycistronic transcripts in other lower metazoan phyla (C.T. Beagley, personal communication, Clary and Wolstenholme 1985; Okimoto et al. 1992). This sequence is 8 bases downstream from the sequence from base 792 to base 837, which has the capacity to fold into a 46-base hairpin. Thus it seems likely that the region between base 787 and base 845 contains the true 3′ end of the lrRNA gene.

Gene Organization. A growing body of work suggests that mitochondrial gene arrangements are likely useful for phylogeny inference. The very small amounts of non-coding mtDNA in metazoans imposes strong constraints upon viable rearrangements, and these changes occur very infrequently (Wolstenholme 1992). That phylogenetic information is present in mtDNA arrangements has been demonstrated on a purely algorithmic basis by Sankoff et al (1992). These arrangements have been used for phylogeny inference in a broad set of metazoan taxa (e.g., Boore et al. 1995, 1998; Smith et al. 1993; Yamazaki et al. 1997). The small section of the sponge mitochondrial genome described here shows features conserved throughout the metazoa.

The precise gene order in this segment of the *Tetilla* sp. mitochondrial genome, lrRNA—COII—tRNA-Lys—ATP8—ATP6, is unknown in any other metazoan mtDNA. However, the organization COII—tRNA-Lys—ATP8—ATP6 is very common in a wide range of mitochondrial genomes. Vertebrate and echinoderm mito-

chondrial genomes show this arrangement directly, while *Drosophila melanogaster* maintains this arrangement but with a tRNA-Asp inserted 3′ to the tRNA-Lys gene. The finding of this gene arrangement in a phylum that is evidently basal to the remainder of the metazoa strongly suggests that this arrangement is ancestral to the metazoa. Nematode mitochondrial genomes do not show this arrangement, although the lrRNA and COII genes are only separated by a single tRNA-His gene. In the earthworm *Lumbricus* (Boore and Brown 1995) the ATP8 and ATP6 genes have become separated by a number of genes, while the COII and ATP8 genes themselves are still separated by only a single tRNA, in this case tRNA-Asp. Similar cases exist in the land snail *Albinaria* (Lecanidou et al. 1994) and the vestimentiferan *Ridgeia* (Wei 1992).

The hexacorallian *Metridium senile* shows an entirely different arrangement of these genes, the only similarity being the contiguous arrangement of the ATP8 and ATP6 genes. In the case of the octocoral *Renilla kolikeri*, however, the arrangement COII—ATP8—ATP6 is found, lacking only the tRNA 3′ to the COII gene. Given the lower degree of sequence divergence between cnidarian and poriferan sequences than between either and any other metazoan phylum, it is interesting that, at least in the case of *Metridium senile*, there may have been less constraint upon genome rearrangements than upon sequence changes. It is worth noting that the *Renilla* mitochondrial genome also encodes a protein which has structural similarity to a bacterial DNA mismatch-repair enzyme (Beagely et al. 1996; Beagely et al. 1995). This gene ordering is likely the case in other octocorals as well (CT Beagley, personal communication). Data from other cnidarian and poriferan mitochondrial genomes will be valuable in determining what kinds of constraints have existed upon genome modification, and whether the very divergent arrangement of the *Metridium senile* mitochondrial genome is unique among Cnidaria.

Conclusion. The mitochondrial genome of this sponge, class Demospongiae, genus *Tetilla*, demonstrates many features typical of other metazoan mitochondrial genomes. In terms of the DNA and amino acid sequences of all of the genes examined, this demosponge mitochondrial genome clusters unequivocally with the rest of the metazoa, further supporting the many results indicating metazoan monophyly. A number of metazoan phylogenies have been produced recently based upon molecular sequence data, with equivocal results regarding the placement of the diploblast phyla relative to the triploblasts and, in particular, with regard to the placement of the sponges relative to the greater metazoan radiation. The results of this study add further support to the inclusion of the Porifera within the metazoa proper. In the case of the *Tetilla* sp. COII DNA and presumed protein sequence, however, the sponges cluster as a monophyletic group with the Cnidaria at very high bootstrap levels. In light of the results of many recent studies which have placed the diploblast phyla as a separate monophyletic metazoan radiation (Christen et al. 1991; Lafay et al. 1992; Kobayashi et al. 1993, 1996: Borchiellini et al. 1998), this result is very interesting.

Both the partial IrRNA gene sequence and the DNA and amino acid sequences of COII show a much higher similarity to cnidarian mitochondrial DNA sequences than to any other metazoan phylum. This result is somewhat surprising given the presumed very ancient divergence times between these phyla and between each and the other metazoan phyla examined. The results suggest that there may have been a higher level of mitochondrial sequence conservation in these two diploblast phyla than there has been in the triploblast phyla.

Note Added in Proof

Our recent unpublished data shows that the arrangement COII–tRNA^{K}–ATP6–ATP8–tRNA^{D}–COIII exists in both *Tetilla* sp. mtDNA and the Ceractinomorph demosponge *Microciona prolifera.* This arrangement of protein coding genes is in common with that of the recently published mtDNA sequence of the Octocoral *Sarcophyton glaucum* [Beaton et al., J Mol Evol 47:697–708 (1998)].

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References

Beagley CT, Macfarlane JL, Pont-Kingdon GA, Okimoto R, Okada NA, Wolstenholme DR (1995) Mitochondrial genomes of Anthozoa (Cnidaria). Prog Cell Res 5:149–153

Beagley CT, Okada NA, Wolstenholme DR (1996) Two mitochondrial group I introns in a metazoan, the sea anemone *Metridium senile:* one intron contains genes for subunits 1 and 3 of NADH dehydrogenase. Proc Natl Acad Sci USA 93:5619–5623

Boore JL, Brown WM (1994) Complete DNA sequence of the mitochondrial genome of the black chiton, *Katharina tunicata.* Genetics 138(2):423–443

Boore JL, Brown WM (1995) Complete sequence of the mitochondrial DNA of the annelid worm *Lumbricus terrestris.* Genetics 141(1): 305–319

Boore JL, Collins TM, Stanton D, Daehler LL, Brown WM (1995) Deducing the pattern of Arthropod phylogeny from mitochondrial DNA rearrangements. Nature 376:163–165

Boore JL, Lavrov DV, Brown WM (1998) Gene translocation links insects and crustaceans. Nature 392:667–668

Borchiellini C, Boury-Esnault N, Vacelet J, Le Parco Y (1998) Phylogenetic analysis of the Hsp70 sequences reveals the monophyly of Metazoa and specific phylogenetic relationships between animals and fungi. Mol Biol Evol 15(6):647–655

Brasier M, Green O, Shields G (1997) Ediacaran sponge spicules from southwestern Mongolia and the origins of the Cambrian fauna. Geology 25(4):303–306

Bridge D, Cunningham CW, Schierwater B, DeSalle R, Buss LW (1992) Class-level relationships in the phylum Cnidaria: evidence from mitochondrial genome structure. Proc Natl Acad Sci USA 89:8750–8753

Bridge D, Cunningham CW, DeSalle R, Buss LW (1995) Class-level relationships in the phylum Cnidaria: molecular and morphological evidence. Mol Biol Evol 12(4):679–689

Christen R, Ratto A, Baroin A, Perasso R, Grell KG, Adoutte A (1991) An analysis of the origin of metazoans, using comparisons of partial sequences of the 28S RNA, reveals an early emergence of the triploblasts. EMBO J 10(3):499–503

Clary DO, Wolstenholme DR (1985) The mitochondrial DNA molecule of *Drosophila yakuba:* nucleotide sequence, gene organization, and genetic code. J Mol Evol 22:252–271

de Bruijn MH (1983) *Drosophila melanogaster* mitochondrial DNA, a novel organization and genetic code. Nature 304(5923):234–241

Degnan B, Degnan S, Naganuma T, Morse D (1993) The ets multigene family is conserved throughout the metazoa. Nucleic Acids Res 21(15):3479–3484

Exposito J, Guellec D, Lu Q, Garrone R (1991) Short chain collagen genes in sponges are encoded by a family of closely related genes. J Biol Chem 266(32):21923–21928

France SC, Rosel PE, Agenbroad JE, Mullineaux LS, Kocher TD (1996) DNA sequence variation provides support for a two-subclass organization of the Anthozoa (Cnidaria) Mol Mar Biol Biotech 5(1):15–28

Gamulin V, Skorokhod A, Kavsan V, Muller IM, Muller WEG (1997) Experimental indication in favour of the introns-late theory: the receptor tyrosine kinase gene from the sponge *Geodia cydonium.* J Mol Evol 44(3):242–252

Gehling J, Rigby J (1996) Long expected sponges from the neoproterozoic Ediacara fauna of South Australia. J Paleontol 70(2):185–195

Higgins DG (1994) CLUSTAL V: multiple alignment of DNA and protein sequences. Methods Mol Biol 25:307–318

Jacobs HT, Elliott DJ, Math VB, Farquharson A (1988) Nucleotide sequence and gene organization of sea urchin mitochondrial DNA. J Mol Biol 202(2):185–217

Kobayashi M, Takahashi M, Wada H, Satoh N (1993) Molecular phylogeny inferred from sequences of small subunit ribosomal DNA, supports the monophyly of the Metazoa. Zool Sci 10:827–833

Kobayashi M, Wada H, Satoh N (1996) Early evolution and the phylogenetic status of diploblasts as inferred from amino acid sequence of elongation factor 1-alpha. Mol Phylogenet Evol 5(2):414–422

Kumar S, Tamura K, Nei M (1993) MEGA: molecular evolutionary genetics analysis, Version 1.0. Pennsylvania State University, University Park

Lafay B, Boury-Esnault N, Vacelet J, Christen R (1992) An analysis of partial 28S ribosomal RNA sequences suggests early radiations of sponges. BioSystems 28:139–151

Leblane C, Boyen C, Richard O, Bonnard G, Grienenberger JM, Kloareg B (1995) Complete sequence of the mitochondrial DNA of the rhodophyte *Chondrus crispus* (Gigartinales). Gene content and genome organization. J Mol Biol 250(4):484–495

Lecandiou R, Douris V, Rodakis G (1994) Novel features of metazoan mtDNA revealed from sequence analysis of three mitochondrial DNA segments of the land snail *Albinaria turrita.* J Mol Evol 38:369–382

Li C, Chen J, Hua T (1998) Precambrian sponges with cellular structures. Science 279:879–882

Liu H (1993) Molecular evolution among several orders of insects based on mitochondrial DNA analysis, PhD thesis, Simon Fraser University, Burnaby, BC

Okimoto R, Macfarlane JL, Clary DO, Wolstenholme DR (1992) The mitochondrial genomes of two nematodes. *Caenorhabditis elegans* and *Ascaris suum.* Genetics 130(3):471–498

Ojala D, Montoya J, Attardi G (1981) tRNA punctuation model of RNA processing in human mitochondria. Nature 290:470–474

Paquin B, Lang BF (1996) The mitochondrial DNA of *Allomyces macrogynus:* the complete genomic sequence from an ancestral fungus. J Mol Biol 255(5):688–701

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence analysis. Proc Natl Acad Sci USA 85:2444–2448

Perez ML, Valverde JR, Batuecas B, Amat F, Marco R, Garesse R (1994) Speciation in the Artemia genus: mitochondrial DNA analysis of bisexual and parthenogenetic brine shrimps. J Mol Evol 38(2):156–168

Pont-Kingdon GA, Beagley CT, Okimoto R, Wolstenholme DR (1994) Mitochondrial DNA of the sea anemone *Metridium senile* (Cnidaria): prokaryote-like genes for tRNA(fmet) and small-subunit ribosomal RNA, and standard genetic code specificities for AGR and ATA codons. J Mol Evol 39:387–399

Pont-Kingdon GA, Okada NA, Macfarlane JL, Beagley CT, Wolstenholme DR, Cavalier-Smith T, Clark-Walker GD (1995) A coral mitochondrial *mutS* gene. Nature 375:109–111

Robitzki A, Schroer H, Ugarkovic D, Pfeifer K, Uhlenbruck G, Muller W (1989) Demonstration of an endocrine signalling circuit for insulin in the sponge *Geodia cydonium.* EMBO J 8(10):2905–2909

Sankoff D, Leduc G, Antoine N, Paquin B, Lang BF, Cedegren R (1992) Gene order comparisons for phylogenetic inference: evolution of the mitochondrial genome. Proc Natl Acad Sci USA 89: 6575–6579

Seimiya M, Ishiguro H, Miura K, Watanabe Y, Kurosawa Y (1994) Homeobox-containing genes in the most primitive metazoa, the sponges. Eur J Biochem 221:219–225

Shenk MA, Steele RE (1993) A molecular snapshot of the metazoan ''Eve.'' Trends Biochem Sci 18:459–463

Simpson TL (1984) The cell biology of sponges. Springer-Verlag, New York, pp. 114–123

Smith MJ, Arndt A, Gorski J, Fajber E (1993) The phylogeny of Echinoderm classes based on mitochondrial gene arrangements. J Mol Evol 36:545–554

Swofford DL (1993) PAUP: phylogenetic analysis using parsimony, Version 3.1.1. Illinois Natural History Survey, Champaign

Wei Y (1992) Gene organization and evolution of mitochondrial genomes from two invertebrate phyla: Vestimentifera and Chaetognatha, PhD thesis. Simon Fraser University, Burnaby, BC

Willmer P (1990) Invertebrate relationships: patterns in animal evolution. Cambridge University Press, Cambridge

Wolff G, Plante I, Lang BF, Kueck U, Burger G (1994) Complete sequence of the mitochondrial DNA of the chlorophyte alga *Prototheca wickerhamii.* Gene content and genome organization. J Mol Biol 237:75–86

Wolstenholme DR (1992) Animal mitochondrial DNA: structure and evolution. Int Rev Cytol 141:173–216

Yamazaki N, Ueshima R, Terrett JA, Yokobori S, Kaifu M, Segawa R, Kobayashi T, Numachi K, Ueda T, Nishikawa K, Watanabe K, Thomas R (1997) Evolution of pulmonate Gastropod mitochondrial genomes: comparisons of gene organizations of Euhadra, Cepaea, and Albinaria and implications of unusual tRNA secondary structures. Genetics 145:749–758