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SPONGE RESEARCH DEVELOPMENTS

# The complete mitochondrial genome of the verongid sponge *Aplysina cauliformis*: implications for DNA barcoding in demosponges

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**Abstract** DNA "barcoding," the determination of taxon-specific genetic variation typically within a fragment of the mitochondrial cytochrome oxidase 1 (*cox1*) gene, has emerged as a useful complement to morphological studies, and is routinely used by expert taxonomists to identify cryptic species and by non-experts to better identify samples collected during field surveys. The rate of molecular evolution in the mitochondrial genomes (mtDNA) of nonbilaterian animals (sponges, cnidarians, and placozoans) is much slower than in bilaterian animals for which DNA barcoding strategies were developed. If sequence divergence among nonbilaterian mtDNA and specifically *cox1* is too slow to generate diagnostic variation, alternative genes for DNA barcoding and species-level phylogenies should be considered. Previous study across the Aplysinidae (Demospongiae, Verongida) family of sponges demonstrated no nucleotide substitutions in the traditional *cox1* barcoding fragment among the Caribbean species of *Aplysina*. As the mitochondrial genome of *Aplysina fulva* has previously been sequenced, we are now able to make the first comparisons between complete mtDNA of congeneric demosponges to assess whether potentially informative variation exists in genes other than *cox1*. In this article, we present the complete mitochondrial genome of *Aplysina cauliformis,* a circular molecule 19620 bp in size. The mitochondrial genome of *A. cauliformis* is the same length as is *A. fulva* and shows six confirmed nucleotide differences and an additional 11 potential SNPs. Of the six confirmed SNPs, NADH dehydrogenase subunit 5 (*nad5*) and *nad2* each contain two, and in *nad2* both yield amino acid substitutions, suggesting balancing selection may act on this gene. Thus, while the low nucleotide diversity in Caribbean aplysinid *cox1* extends to the entire mitochondrial genome, some genes do display variation. If these represent interspecific differences,

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then they may be useful alternative markers for studies in recently diverged sponge clades.

**Keywords** mtDNA · Porifera · Demospongiae · Verongida

**Abbreviations**

| | |
|---|---|
| mtDNA | Mitochondrial genome |
| *atp6*, *8*, *9* | ATP synthase F0 subunit # |
| *cob* | Apocytochrome b |
| *cox1-3* | Cytochrome *c* oxidase # |
| *nad1-6*, *4L* | NADH dehydrogenase subunit # |
| *rnS* | Small ribosomal RNA |
| *rnL* | Large ribosomal RNA |

## Introduction

"DNA barcoding," whereby the nucleotide sequence of a genomic fragment is utilized to aid the identification of an organism (Hebert et al., 2003), has emerged as a complementary approach to purely morphologically based taxonomic studies, and has proven especially useful in helping experienced taxonomists uncover cryptic species and for generalist researchers, such as community ecologists, to better identify organisms during broad surveys of biodiversity. DNA barcoding traditionally utilizes a 710-bp stretch at the 5′ end of the mitochondrial cytochrome oxidase I (*cox1*) gene. This fragment is relatively easy to amplify by PCR because of primer regions conserved across metazoan phyla (Folmer et al., 1994) and the abundance of mitochondrial DNA in eukaryotic cells. Mitochondrial genes are also preferred for such studies because of the relatively high rate of sequence evolution in bilaterian animals (especially at third-codon positions) which allows for discrimination even between recently diverged species, rapid coalescence, and because mitochondrial genomes (mtDNA) are typically maternally inherited and do not recombine (Boore & Brown, 1998; but see Galtier et al., 2009). The utility of DNA barcoding promises to advance organismal and ecological studies across the metazoan tree as the catalogs of taxon-specific variation are further expanded (Miller, 2007).

The Sponge Barcoding Project (Wörheide & Erpenbeck, 2007) has made the case for implementing DNA barcoding in poriferan research. From a morphological perspective, sponges are generally a character-poor group of organisms. Most taxonomy is based on the types and arrangements of siliceous and calcareous spicules, although the differences between species are often subtle, or relate to factors such as spicule size that could potentially be affected by the environment (e.g., Maldonado et al., 1999). The lack of characters has led, in part, to the presence of long-ranging "cosmopolitan" species that likely represent a number of cryptic species (Klautau et al., 1999). Further, the paucity and plasticity of characters make correct identification difficult for non-specialists. Indeed, in a survey of 138 faunal inventories from the North Atlantic, Schander & Willassen (2005) found that sponges were identified to the species level in less than half the cases, in contrast to groups like annelids and mollusks for which more than 75% of specimens were identified to species level. Given the importance of sponges to the discovery of new bioactive compounds (Sipkema et al., 2005) and the ecological importance of sponges in essentially every marine and aquatic environment (Bell, 2008), a tool for the rapid identification of specimens by natural products researchers and community ecologists would be of high value.

Initial studies using DNA barcoding in sponges, however, have seen mixed results. *Cox1* successfully discriminates between species in the genus *Tethya* (Heim et al., 2007a) and *Scopalina* (Blanquer & Uriz, 2007). In other cases, though, species that are clearly discriminated on the basis of morphology show the same *cox1* haplotype (Schroder et al., 2003; Heim et al., 2007b; Pöppe et al., 2010). Studies of *cox1* intraspecific variation in sponges also show extremely low rates compared to those in bilaterian species-level populations (Duran et al., 2004; Wörheide, 2006). Xavier et al. (2010) found high intraspecific divergences within the species *Cliona celata* but suggested this was evidence for cryptic speciation. The low variation in species-level *cox1* sequences is likely a direct reflection of the overall low rates of molecular evolution in sponge (and more generally nonbilaterian) mitochondrial genomes (Shearer et al., 2002; Lavrov et al., 2005; Huang et al., 2008). This rate difference between the nonbilaterian animals (sponges, cnidarians, and placozoans) and bilaterians is so drastic that phylogenetic trees built from complete mitochondrial genomes result in an artifactual (see Sperling et al., 2009) "Diploblastica," where the fast-evolving bilaterians are attracted toward outgroups and the

metazoan root is mis-placed between the slow-evolving sponges, placozoans, and cnidarians and the fast-evolving bilaterians (reviewed by Lavrov, 2007). The slow rate of mitochondrial sequence evolution in sponges and the inability to discriminate between species has led to the suggestion that the standard 5′ *cox1* barcode may not be suitable for DNA barcoding in many sponge taxa (Pöppe et al., 2010), as well as to a search for alternative markers, such as the 3′ end of *cox1* which may have more phylogenetic signal (Erpenbeck et al., 2005). It is worth noting that although DNA barcoding as a tool (e.g., specimen identification by non-specialists) can be used in a non-phylogenetic context, slow mitochondrial evolution (and consequent lack of signal) is a problem for resolution of species- and genus-level phylogenies using standard mitochondrial markers as well.

Even though nonbilaterian mitochondrial genomes exhibit low rates of molecular evolution, mitochondrial genes remain prime targets for barcoding markers and species-level phylogenetic questions given their clonal inheritance, lack of recombination, and perhaps most importantly the abundance of mtDNA in a cell, leading to relatively easy amplification even from sub-optimal specimens, for instance, those in museum collections. The question remains whether faster-evolving gene regions can be identified for use in conjunction with the standard 5′ *cox1* barcode. To test whether the strong conservation of *cox1* extends over the entire mitochondrial genome, and to aid the search for other potential mitochondrial markers, we sought to examine variability in the mitochondrial genomes of two closely related congeneric species of Demospongiae. We focused on the genus *Aplysina* Nardo 1834 (Demospongiae, Verongida, Aplysinidae), which is a genus of aspiculate sponges within the Myxospongiae or "G2" clade (Borchiellini et al., 2004; Nichols, 2005; Lavrov et al., 2008; Sperling et al., 2009). The taxonomy and phylogenetic relationships of the Aplysinidae have been well studied from both a morphological and molecular perspective in recent years, making it in some sense a "test case" for species discrimination within Porifera (Schmitt et al., 2005; Erwin & Thacker, 2007; Heim et al., 2007b; Kloppell et al., 2009; Lamarao et al., 2010). Importantly, despite the Caribbean *Aplysina* species showing clear morphological differences (e.g., Erwin & Thacker, 2007), they show no variation in the standard 5′ *cox1* fragment at the nucleotide level (Heim et al., 2007b).

The complete mitochondrial genome of one species of *Aplysina*, *A. fulva*, has already been sequenced (Lavrov et al., 2008), and thus we sequenced the mitochondrial genome of its likely sister species, *A. cauliformis* (Erwin & Thacker, 2007). The mitochondrial genomes of congeneric species of homoscleromorph sponges (classically considered demosponges) have been sequenced, but this sponge clade is now regarded as a separate sponge lineage (Borchiellini et al., 2004; Sperling et al., 2007, 2009; Lavrov et al., 2008). Congeneric species of the demosponge genus *Amphimedon* also have sequenced mitochondrial genomes, but that genus is unlikely to be monophyletic (Lavrov et al., 2008). Thus, this study on *Aplysina* represents the first comparison of mitochondrial genomes from closely related species in a monophyletic demosponge genus. Given the extremely recent divergence of Caribbean *Aplysina* species inferred by Heim et al. (2007b), this probably represents one of the most severe possible cases for molecular discrimination of species. Consistent with other reports of mitochondrial DNA variation in other sponges, we observe only six confirmed nucleotide substitutions (and 11 other sites with potential variation) between these two species. Of the six SNPs that can be confidently assigned, two are found in *nad5* and two in *nad2*. Both *nad2* variants result in amino acid changes, suggesting that exploration of that gene as a marker for species-level discrimination may be warranted.

## Materials and methods

### Collection and amplification

*Aplysina cauliformis* was collected using SCUBA from the M1 reef at the Discovery Bay Marine Station, Jamaica at 15 m depth. Tissue from one individual was lysed in 8 M urea buffer (Chen & Dellaporta, 1994), incubated at 65°C for 20 min, and total DNA was prepared by extraction in phenol–chloroform (1:1) and precipitation in 0.7 volumes isopropanol. To confirm the identity of the sponge, the 28S-ITS2 fragment used by Erwin & Thacker (2007) was also sequenced using the protocol outlined in their article, and submitted to GenBank (HQ730891). Phylogenetic analysis of that fragment demonstrated that the sponge was correctly identified (Supplemental Figure 1). A partial fragment of *cox1* was then amplified by PCR using the primers

LCO 1490–HCO 2198 (Folmer et al., 1994), and a partial fragment of *rnL* with custom primers CGAGA AGACCCCATTGAGCTTTACTA and TACGCTGT Sequence-specific primers pairs (CGAGAAGACC CCATTGAGCTTTACTG/CATATCTACCGAACC CCCAGAATGTG and CGCCCCAACTAAACTGT CTGCTTTAC/TATCTGCACCAGGCTCAATGTT AGGA) were then designed and used with Takara LA Taq for long-range PCR. Cycling conditions were as follows: a 1-min initial denaturation at 94°C, 30 cycles of 98°C for 10 s, and 62°C for 12 min but adding 10 s to the extension time every cycle after the first ten cycles, and a final extension step at 72°C for 10 min. This produced two overlapping fragments of 8 and 12 kilobases (kb), respectively. These products were gel-purified, sheared by sonication, and end-repaired with the DNA Terminator kit (Lucigen). Two–four kb fragments were size-selected by gel electrophoresis, blunt-end cloned into the pSmart LC-Kan vector (Lucigen), and transformed into *E. cloni* Supreme cells (Lucigen). Colonies were screened by PCR for the presence of inserts using flanking vector primers SL1 and SR2 (Lucigen). Sixty PCR products were purified by poly-ethylene glycol–NaCl (PEG:-NaCl) (Lis & Schleif, 1975) and sequenced by BigDye® Terminator v3.1 cycle sequencing on ABI PRISM® 3730 DNA Analyzers (Applied Biosystems, Inc.) at the DNA Analysis Facility on Science Hill, Yale University.

### Sequence assembly and annotation

Sequences were assembled from chromatography data using the Phred Phrap Consed software package release 15.0 (Ewing & Green, 1998; Gordon et al., 1998). Regions of lower quality data were sequenced by direct PCR on genomic DNA, or additional sequencing of select gap spanning clones. The resulting 19620-bp contig had a minimum of 2–8× coverage on both strands with high phred values (40 or greater), with the exception of 2.6% of the genome that had 1× coverage with high phred values (GenBank: EU518938). The suite of tRNA genes were identified by tRNAscan-SE (Lowe & Eddy, 1997) using the program's default parameters for mitochondria or chloroplast sequences and the Mold and Protozoan mitochondrial translation code. Protein- and RNA-coding genes were initially identified using the program DOGMA (Wyman et al., 2004), and confirmed and annotated by Blast2 (Tatusova & Madden, 1999) and ClustalW (Thompson et al., 1994).

## Results

### Gene content

The complete mitochondrial genome (mtDNA) of *A. cauliformis* was shotgun-sequenced and assembled as a 19620-bp circular molecule. This sequence was nearly identical to that of *A. fulva* reported by Lavrov et al. (2008), but owing to the number of mitochondrial genomes reported in that study, detailed features of each individual mtDNA could not be reported, and thus we provide an in-depth examination of the *A. cauliformis* genome here. Analysis of the complete mitochondrial genome sequence predicted 14 protein-coding genes, 2 ribosomal RNA genes, and 25 transfer RNA genes. Seven of the protein-coding genes, both rRNAs, and 12 of the tRNAs were coded on one strand (top), while the remaining genes and tRNAs were arranged in a contiguous inverted block on the reverse strand. The protein-coding gene complement included 11 of the respiratory genes (*cob*, *cox1-3*, *nad1-4*, *4L*, *5*, *6*) canonical to metazoan mtDNA, as well as the ATP synthase F0 subunits 6, 8, and 9 (*atp6*, *atp8*, *atp9*) (Fig. 1) found in most demosponge mitochondrial genomes. As seen in most other demosponge mitochondrial genomes (Lavrov, 2007) *A. cauliformis* has no additional genes, large introns, or open reading frames of unknown function, or programmed translational frameshifting as seen in hexactinellid sponge mitochondrial genomes (Rosengarten et al., 2008). All of the protein-coding genes were found to contain a canonical AUG start codon, except *nad6* which appeared to employ a GUG codon 54 bases upstream of the first AUG, and all coding sequences terminated translation with the UAA codon except for *atp8* and *nad4L*, which use UAG.

The 25 tRNAs of *A. cauliformis* (Supplemental Figure 2) included two isoacceptors for methionine, the initiator $trn^{Met}(cau)i$ and the elongator $trn^{Met}(cau)e$ (*Mi* and *Me*, respectively, on Fig. 1). The following criteria were used to distinguish between the initiator and elongator *trnM* genes: Initiator $tRNA^{Met}$ molecules lack Watson–Crick base-pairing at the 1:7# position, have a R11:Y2# pair, and have a series of three guanines in the anticodon stem (Drabkin et al.,

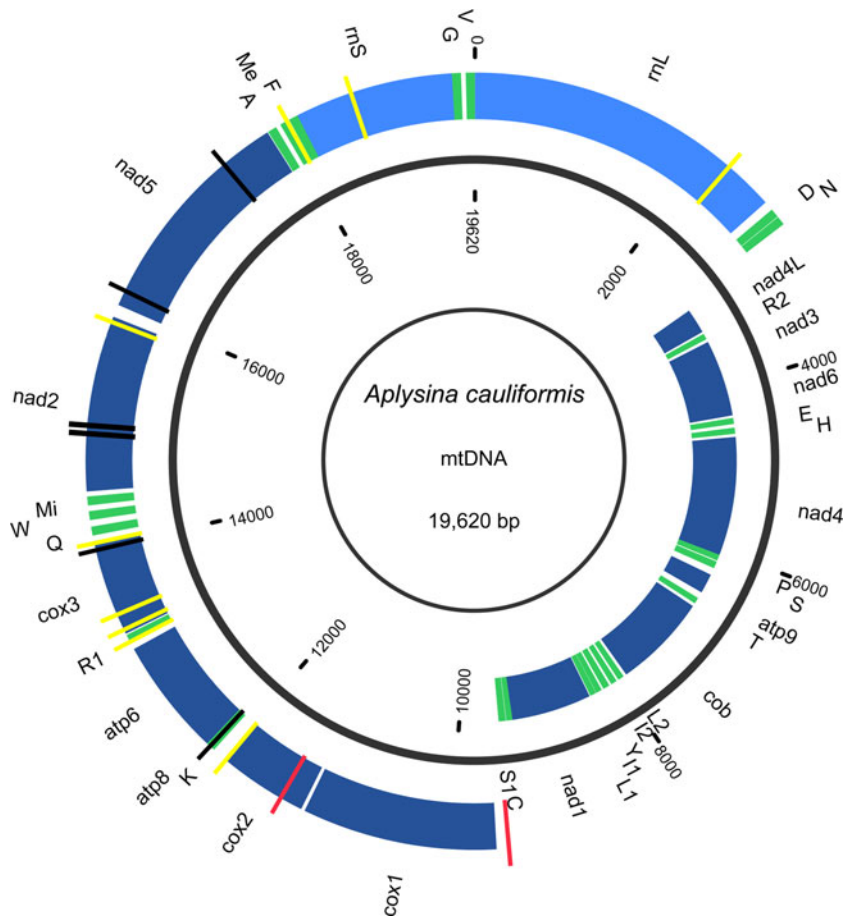


**Fig. 1** *Aplysina cauliformis* mtDNA. The mitochondrial genome of the demosponge *Aplysina cauliformis* was determined to be a 19620-bp circular molecule encoding 14 respiratory genes, two rRNAs and 25 tRNAs. Protein-coding genes are represented with dark blue boxes, ribosomal RNAs in light blue, and tRNAs in green. The tRNAs are labeled with their one letter IUPAC amino acid abbreviation. Genes on the outside of the circle are transcribed clockwise, on the top strand. Genes inside the circle are transcribed counter-clockwise, on the reverse strand. The two isoacceptors for methionine are labeled *Mi* and *Me* for the initiator and elongator *trnM*, respectively. All other tRNA isoacceptors are enumerated 1 and 2. Confirmed nucleotide substitutions are indicated by black bars, the unresolved *A. fulva* positions (base N) are indicated by yellow bars, and the possible SNPs with conflicting sequence reads are red. The mtDNA map was generated with Circos (Krzywinski et al., 2009)

1998; Stortchevoi et al., 2003). Based on these motifs, the *trnM* at position 14363 was designated as the initiator, and the one at position 17987 was designated as the elongator. A third *trnM* gene was predicted by tRNAscan-SE on the reverse strand at position 8096, but was annotated as an isoacceptor for isoleucine according to the convention established for demosponge mtDNA (Lavrov et al., 2005). This annotation strategy assumes the *trnM* undergoes secondary modification of the C34 base in the anticodon to lysidine (2-lysyl-cytidine), thus gaining specificity for isoleucine as $tRNA^{Ile}(cau)$ (*I2* on Fig. 1). Evidence for conversion of *trnM* to *trnI* has been obtained for bacteria lacking a *trnI*, as well as in the potato mitochondrion, by both molecular and biochemical assays (Weber et al., 1990; Muramatsu et al., 1998). However, such experimental confirmation of lysidinylation is still lacking in sponges, and thus this annotation should be taken with some degree of caution.

In addition to methionine and isoleucine, leucine, arginine, and serine were also found to have two isoacceptor tRNAs (*L1*, *L2*, *R1*, *R2*, *S1*, and *S2* in Fig. 1). Several tRNAs had remarkable structural

features. Both *trnS* genes and the *trnY* gene revealed long variable arms, diagnostic for type-2 tRNAs (Lavrov et al., 2005). *trnA* had a mismatch in its anticodon arm, while *trnC*, *F*, *H*, *I1*, *P*, and *R1* all revealed mismatches in their acceptor arm. *trnL2* had a two pair mismatch—U1:U71 and U2:U72—at the top of its acceptor arm (Supplemental Figure 2). These mismatches are frequently seen in the primary sequence of animal mitochondrial tRNAs, and are thought to be corrected by an RNA editing process (Lavrov et al., 2000).

### Comparison to *A. fulva*

The mitochondrial genome of *A. cauliformis* is nearly identical to that of *A. fulva*, on both a structural level and at the nucleotide and amino acid level. Indeed, both genomes are the same length (19620 bp) and only six single nucleotide differences were confirmed between them (Fig. 1; Table 1). Of these six, three represented transitions and three transversions. One resided in the trnK but did not result in a change in isoacceptor. The remaining five variants were located in the protein-coding genes *cox3*, *nad2*, and *nad5* (Table 1). The entire *cox1* gene, in addition to the traditional 5′ barcoding fragment investigated by Heim et al. (2007b), was therefore found to be invariant between the two species. Four of the nucleotide changes in protein-coding genes represented silent substitutions, while two resulted in changes at the amino acid level in *nad2*. Two other potential variants (positions 9537 and 11443) were identified but have conflicting chromatogram traces. An additional nine differences (at nucleotide positions 2221, 11979, 13208, 13317, 13450, 14054, 15860, 18046, and 18608) correspond to ambiguous bases (N's) in the *A. fulva* genome sequence (EU237476.1) and thus cannot be evaluated at present.

**Table 1** Summary of nucleotide variation between *Aplysina cauliformis* and *A. fulva*

| Position | Locus[a] | Nucleotide: *cauliformis/fulva*[b] | Silent/ substitution[c] |
|---|---|---|---|
| Confirmed | | | |
| 12147 | *trnK* | A/G | |
| 13996 | *cox3* | T/A | Silent |
| 14932 | *nad2* | A/T | L/I |
| 14941 | *nad2* | C/T | F/L |
| 16133 | *nad5* | C/T | Silent |
| 17432 | *nad5* | C/A | Silent |
| Unresolved *fulva* base N | | | |
| 2221 | *rnL* | G/N | |
| 11979 | *cox2* | T/N | |
| 13208 | *trnR* | T/N | |
| 13317 | *cox3* | T/N | |
| 13450 | *cox3* | A/N | |
| 14054 | *cox3* | T/N | |
| 15860 | *nad2* | T/N | |
| 18046 | *trnMe* | T/N | |
| 18608 | *rnS* | A/N | |
| Conflict | | | |
| 9537 | IG | A or G/A | |
| 11443 | *cox2* | A or G/A | K/E |

[a] IG refers to intergenic sequence

[b] For each instance of nucleotide variation, the *A. cauliformis* base is listed first, followed by the *A. fulva* base. For the "read conflicts," i.e., the cases in which the *A. cauliformis* chromatograms are inconsistent, both possible bases are listed

[c] When nucleotide variation leads to changes in amino acid sequence, the *A. cauliformis* amino acid is listed first, followed by the corresponding residue in the *A. fulva* gene product

## Discussion and conclusions

Comparison of the complete *A. cauliformis* and *A. fulva* mitochondrial genomes, the first congeneric mitochondrial genomes sequenced within the Demospongiae sensu stricto, demonstrates that the rate of molecular divergence is exceedingly low across the entire mtDNA. Only six confirmed nucleotide differences, encoding two predicted amino acid changes, and no structural variation, were identified between these molecules. These complete mitochondrial genomes are so similar that the possibility should remain open that these two forms are in fact the same species. Nevertheless, we follow in this study previous researchers who have found small but consistent morphological (Erwin & Thacker, 2007) and molecular (Erwin & Thacker, 2007; Lamarao et al., 2010) differences between the Caribbean aplysinids and suggested that these be retained as separate species (see also discussion in Schmitt et al., 2005). Further, as only one individual from each species was compared in this study, the comparisons must be viewed with some degree of caution. Specifically, it cannot be determined whether the six observed nucleotide

differences are synapomorphies shared by all members of a given species, or whether they are autapomorphic changes characterizing a specific population. Future studies comparing the complete mitochondrial genomes from multiple individuals in different species will be needed to see if these characters truly delineate the two species.

The entire *cox1* gene, including the 5′ and 3′ flanking sequences, is monomorphic and uninformative between the two species. Our results are in accordance with the minimal *cox1* variation observed between sponge genera (and among other nonbilaterian animals in general; Shearer et al., 2002; Schroder et al., 2003; Heim et al., 2007b; Huang et al., 2008; Pöppe et al., 2010). This overall low level of sequence variation poses a general problem for species discrimination and mitochondrial DNA barcoding within the demosponges.

One solution to the paucity of informative sites in the *cox1* sequence traditionally employed both in barcoding studies and in species- and genus-level phylogenies is to compare other genes. Barcoding studies (and species-level phylogenies in general) have often focused on the use of mitochondrial genes because of the advantages listed above, such as high copy number and uniparental inheritance, but they need not be limited to these genes. The 28S-ITS2 fragment used in the study of Erwin & Thacker (2007), for instance, is not invariant and shows three nucleotide substitutions between the *A. cauliformis* sequenced here and the available *A. fulva* sequences. Thus, while resolution remains low, this fragment does appear to better discriminate between aplysinid species (see the results of that study and Supplemental Figure 1). The future use of these ribosomal genes, introns in protein-coding genes and microsatellite studies may all prove beneficial for demosponge species discrimination and in species-level phylogenies. A comparative review of these genes, however, is outside the scope of this article, and therefore we restrict the discussion to the lessons that might be gained from the variation present in the congeneric mitochondrial genome comparison conducted here, with the caveat that comparison of multiple individuals from each species would give a fuller picture of variation.

The variation observed between *Aplysina* species was confined to single nucleotide variation detected in *trnK*, *nad2*, *nad5*, and *cox3*. In particular, both SNPs in *nad2* cause amino acid substitutions, suggesting balancing selection may contribute to variation in this gene. This hypothesis would predict more widespread variation within populations and between sponge species might exist. Studies of selection acting on the mtDNA of bilaterian species, such as *Drosophila melanogaster*, infer that purifying selection eliminates most novel mutation in these molecules (Haag-Liautard et al., 2008). Our hypothesis of a potential signature of balancing selection in *nad2* does not preclude the possibility that purifying selection eliminates most variation in the rest of the mtDNA.

The mitogenomic location of observable variation in *Aplysina* is congruent with the survey of Wang & Lavrov (2008) who calculated the rate of molecular evolution for protein-coding genes across all published demosponge mitochondrial genomes. They found that *atp8* and *nad6*, both relatively small genes on the order of 77 and 190 predicted amino acids for *Aplysina*, respectively, evolved considerably faster than all other genes. Of the larger genes, the two fastest-evolving genes were *nad2* and *nad5*, both of which show variation in our comparison of *Aplysina* species. In contrast, *cox1* showed the lowest rate of molecular evolution across all genes in the mitochondrial genome, and no variation between the two *Aplysina* species.

The results of this congeneric study, combined with the more global survey of Wang & Lavrov (2008) and other studies of DNA barcoding in demosponges (e.g., Pöppe et al., 2010), suggest that while *cox1* will be useful in resolving relatively ancient species-level divergences, its utility is greatly diminished for recognizing recently diverged sponge species, even when the more variable 3′ region is included. The fastest-evolving genes in demosponge mitochondrial genomes (Wang & Lavrov, 2008), *atp8* and *nad6*, are relatively small, and alignment across demosponges does not reveal any conserved regions within these genes that could be used to build universal degenerate primers. Thus, while primer regions that will work across all sponges are unlikely to be found for *atp6* and *atp8*, with the sequencing of more complete mitochondrial genomes, the possibility of clade-specific targeted primers remains open (e.g., Xavier et al., 2010). Alignments of *nad2* and *nad5*, on the other hand, show several variable regions separated by conserved regions where primers could be placed. Given their relatively higher rates of molecular evolution, these two genes, perhaps in conjunction with ribosomal genes or microsatellite studies, warrant

exploration as a potential complement to the traditional *cox1* gene for mitochondrial DNA barcoding and for species-level phylogenetics in closely related demosponge species.

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