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Research paper

Fast fish face fewer mitochondrial mutations: Patterns of *dN*/*dS* across fish mitogenomes

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ABSTRACT

Mitochondrial DNA is routinely used to answer a variety of biological questions; and there is growing evidence suggesting that its accumulation of mutations is influenced by life history, effective population size and cellular energy requirements. This study examines the influence of phylogenetic patterns of metabolic activity on the evolution of mitochondrial DNA in fishes, given energy requirements associated with high performance versus sedentary life histories. It was determined that all 13 protein coding genes of the mitogenome experience a relaxation of purifying selection in sedentary fishes. This phenomenon was not detected in nuclear housekeeping genes, suggesting that it can be explained by the energy requirements of these groups, and possibly their effective population sizes. This study also examined the subunit binding sites of two subunits of cytochrome c oxidase (*COXI* and *COXIII*), and did not detect any differences in selection between these groups of fishes. These cytochrome c oxidase subunits interact with subunits that are encoded by the nuclear genome and it has been suggested that a unique form of coevolution occurs between these genomes in order to maintain function, and may have implications for speciation. Although this was not a main focus of this study, our preliminary results suggest that substitutions in subunit binding site regions are rare. The results from this study add to the growing literature on the complex relationship between mitochondrial DNA and the evolution of life histories across the tree of life.

1. Introduction

Mitochondria contain unique genomic material which is routinely used to examine biological relationships within and between species. Understanding the forces that shape the evolution of mitochondrial DNA provides important contextual information, because this evolution is influenced by a wide variety of factors, which in turn, impact the information content of this genome. As the aerobic site of cellular energy production in eukaryotes, mitochondria generate ATP through the biochemical process of oxidative phosphorylation (OXPHOS). Most of the genes encoding protein subunits involved in OXPHOS have been incorporated into the nuclear genome, leaving only 13 core subunits encoded by the mitochondrial genome (mitogenome). Mitogenomes, while initially thought to evolve neutrally (reviewed by William et al. (1995)), are not only influenced by purifying selection, but also evolve adaptively (Blier et al, 2001; William et al., 2005; da Fonseca et al., 2008; Castoe et al., 2009; Shen et al., 2010; Menezes et al., 2013). Additionally, the evolutionary rate of mitochondrial DNA is highly variable across the tree of life (Martin et al., 1992; Nabholz et al., 2008); and highly active versus sedentary species are distinct candidates for comparing differences in mitogenome evolution. Because energy production is considered of critical importance for "high performance" life history strategies, OXPHOS subunits are thought to be under strict functional constraints (Dalziel et al., 2006; Sun et al., 2011). Conversely, as high energy production is considered less critical for sedentary species, a relaxation in purifying selection has been suggested (Shen et al., 2009; Chong and Mueller, 2013). Consequently, presumed fitness losses from non-synonymous mutations are predicted to result in high-performance species having lower ratios of non-synonymous to synonymous mutations (*dN*/*dS*) relative to sedentary species (Shen et al., 2009; Sun et al., 2011).

Work by Shen et al. (2009) and Sun et al. (2011) examined *dN*/*dS* variation in flightless and flying birds, mammals, and migratory and non-migratory fish by exploring patterns of purifying or positive

Abbreviations: OXPHOS, oxidative phosphorylation; Mitogenome, mitochondrial genome; *dN*/*dS*, ratio of non-synonymous to synonymous mutations; PIC, phylogenetically independent contrasts; *PLAGL2*, *Pleiomorphic adenoma gene-like 2*; *RIPK4*, *Receptor-interacting serine-threonine kinase 4*; *ZIC1*, *Zic family member 1*; GARD, Genetic Algorithm Recombination Detection; REV, General Reversible Model; Branch-site REL, Branch-site random effects likelihood; MEME, Mixed effects model of evolution; R = *Ts*/*Tv*, transitions and transversions ratio; ω *dN*/*dS*, The hypothesis testing software package used in this study is called RELAX but that is not an acronym; *COXI COXIII*, *cytochrome c oxidase subunits I and III*; RFLP, restriction fragment length polymorphism; N_e, effective population size; ROS, reactive oxygen species.

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selection. Purifying selection is defined by a *dN*/*dS* ratio lower than one, as it tends to remove non-synonymous variation from a population; positive selection is defined by a *dN*/*dS* ratio greater than one, as it tends to promote non-synonymous variation in a population; and a ratio of 1 indicates neutrality (Graur and Li, 2000; Wertheim et al., 2014). Shen et al. (2009) sorted bird and mammal species into relatively active and inactive groups based on maximum traveling speeds divided by body length. Sun et al. (2011) sorted fish based solely on whether the species exhibit migratory behavior. They both found that species in highly locomotive groups showed lower *dN*/*dS* ratios (indicative of purifying selection) when compared to weakly locomotive species. Furthermore, Shen et al. (2009) concluded that OXPHOS genes of weakly locomotive mammals and birds showed slightly higher *dN*/*dS* ratios, a pattern sometimes characterized as 'relaxed purifying selection'. This is generally defined as a reduction in selection intensity against substitutions in one group of organisms when compared with another (for a more detailed discussion see: Wertheim et al. (2014)). While insightful, both studies possess methodological issues. For Shen et al. (2009) the *dN*/*dS* calculations were guided by phylogenies generated from their mitochondrial dataset. Also, these analyses were parsed into smaller subsets – consequently, an inclusive phylogeny and therefore a single analysis spanning any one taxon (mammal or bird) could not be used. Additionally, phylogenetically independent contrasts (PIC) were performed to allow the use of parametric statistics on their dataset (Felsenstein, 1985). This is considered an incorrect application of PIC due to a number of factors, the main one being that *dN*/*dS* values are already a form of contrast calculated between two branches (Popadin et al., 2007). Of direct relevance to this study, Sun et al. (2011) explored our same question, regarding purifying selection based on energetics, by computing *dN*/*dS* ratios for migratory and non-migratory fish. However, Sun et al. (2011) only used parametric statistics (t-tests and ANOVA) in their comparative analyses, which are confounded by the nature of genomic data which are neither independent, nor normally distributed.

Here, we explore patterns of synonymous and non-synonymous substitutions across mitogenomes of fish species with high and low energy requirements, and expand upon the work of Shen et al. (2009) and Sun et al. (2011); we do this by using the current phylogenomic methods to compare *dN*/*dS* ratios across all 13 protein coding genes in mitogenomes from fishes with high performance and sedentary lifestyles. The term "high performance" is often used to refer to the tunas, billfishes, and their relatives (Dewar and Graham, 1994; Brill, 1996; Little et al., 2012) due to their streamlined bodies, high swimming speed, high metabolic rates, endothermic thermoregulation, and gills and muscle tissues packed with mitochondria (Bushnell and Jones, 1994; Brill, 1996; Graham and Dickson, 2000, 2004; Bernal et al., 2001; Block et al., 2001). The sedentary group of fish for this study will be sit-and-wait predators that inhabit both shallow and deep water environments (selection for both groups is further explained in Section 2).

While the first focus of this study explores *dN*/*dS* ratios of the mitogenome, the manner in which its proteins interact with those encoded by the nuclear genome is another interesting avenue of research. The holoenzyme assembly of OXPHOS proteins relies on the biochemical and structural properties of subunit binding/interaction sites. The OXPHOS enzymes are large, and comprised numerous subunits, and these subunits are encoded by both the mitogenome and the nuclear genome. This results in a unique form of co-evolution known as mitonuclear evolution, which acts to maintain the structure and function of OXPHOS proteins (Rand et al., 2004; Niehuis et al., 2008). It is worth stating that this form of co-evolution could occur between other protein domains encoded by the mitogenomes; and the binding sites are an excellent candidate to explore this interaction due to their proximity and functional importance. These subunit binding sites are highly conserved across all aerobic taxa because of their critical functions (Schmidt et al., 2001). Relatedly, there is interesting observational and experimental evidence from cytoplasmic hybrid (cybrid) crosses to suggest that mutations in OXPHOS subunit binding sites can drive speciation events if one population gains a non-synonymous substitution in the nuclear genome, while others fail to acquire a compensatory mutation in the mitogenome (Mckenzie et al., 2003; Harrison and Burton, 2006; Osada and Akashi, 2011). It is possible that a relaxation of purifying selection in sedentary fishes may also be detectable in these mitochondrial subunit binding sites, with interesting implications for speciation and physiology.

Given the tantalizing hypotheses that can be formed once *dN*/*dS* ratios are observed, the main question of this study is: "Do sedentary fish experience a relaxation of purifying selection in their mitogenomes compared to high performance fish?" Here, we will explore this question at three scales: 1) protein coding genes throughout the mitogenome; 2) within a subset of these genes, at OXPHOS subunit binding sites in the mitogenome; and 3) a selection of nuclear housekeeping genes, not explicitly involved in energy production.

2. Methods

2.1. Taxon selection

We considered mitogenomes available for tunas (Scombridae) and billfishes (Xiphiidae and Istiophoridae) as being in a high performance group. Also, to increase the phylogenetic sample size, mackerels (Scombridae), kingfish (Carangidae), jacks (Carangidae), and the opah (*Lampris guttatus*) were included in this group, because they share many physiological and life history traits with the tunas and billfish (Sepulveda and Dickson, 2000; Clark and Seymour, 2006; Dalziel et al., 2006; Polovina et al., 2007; Runcie et al., 2009). The fishes we consider in the sedentary group are the anglerfishes (Lophiiformes), eels (Anguilliformes, Saccopharyngiformes, Notacanthiformes, Synbranchiformes), whalefish (Cetomimiformes), and brotulas (Ophidiiformes) (Retzer, 1991; Nelson, 1994; Pietsch, 2009; Vieira et al., 2012). Anglerfish, for example, epitomize the sedentary sit-and-wait lifestyle, as their swimming musculature is extremely reduced and their fins are not effective for prolonged swimming (Moore, 2002; Pietsch, 2009; Luck and Pietsch, 2008). A list of all species, and their group affiliations are provided in Supplementary List 1.

2.2. Data mining

We examined all 13 OXPHOS genes from a total of 48 mitogenomes from ray finned fishes (Actinopterygii) with high performance ($n = 22$) or sedentary ($n = 26$) lifestyles (Supplementary List 1). For each species, the complete mitogenome Genbank record files were downloaded in December, 2013, and each of the 13 OXPHOS genes were extracted and aligned using Genbank-to-TNT (Goloboff and Catalano, 2012) (Genbank accession numbers for all genes used are listed in Supplementary Lists 1 and 4). These were further aligned in MEGA6 (Tamura et al., 2013) using ClustalW and fine tuned by eye to ensure that all genes were in the correct reading frame. All sequences for NADH dehydrogenase subunit 6 were reverse complimented due their encoding by the reverse strand of the mitogenome.

In order to avoid the circular logic associated with computing a phylogeny and using it to guide *dN*/*dS* computations from the same data, we used the topology from the recent, and taxonomically extensive, fish phylogeny by Betancur-R et al. (2013). This new classification of bony fishes is based on a maximum likelihood estimation using 20 nuclear genes, and mitochondrial 16S sequences from 1416 species. We used the published treefile of Betancur-R et al. (2013), downloaded from the Dryad Digital Repository, and parsed it using Archaeopteryx (Han and Zmasek, 2009), to only include the taxonomic groups relevant for this study (See Supplementary List 2 for all taxa that were used from Betancur-R et al. (2013)). For the 48 species examined here, there were 15 cases where the species in the phylogeny did not match the species of available mitogenomes (Supplementary List 3), and in these

instances, we paired the species in the phylogeny with the available mitogenome of a congener.

To test whether sedentary fishes' nuclear genes experience relaxation of purifying selection, similar to their mitochondrial genes, we chose three housekeeping genes. These nuclear genes are: Pleiomorphic adenoma gene-like 2 (*PLAGL2*), Receptor-interacting serine-threonine kinase 4 (*RIPK4*) and Zic family member 1 (*ZIC1*). The majority of these nuclear loci were generated by Betancur-R et al. (2013) with a few being generated by Near et al. (2012). These nuclear loci were not available for all taxa, but enough were present for all the major lineages (*PLAGL2* n = 37, *RIPK4* n = 33, *ZIC1* n = 37). Please consult Supplementary List 4 for associations between nuclear genes and species.

2.3. Analysis

In order to examine biases between transitions and transversions (R = *Ts*/*Tv*) in the mitogenome dataset, TREE-PUZZLE (Schmidt et al., 2002) was used with default settings as per Batista et al. (2011). Because nucleotide substitution models are sensitive to recombination, Genetic Algorithm Recombination Detection (GARD) was used to check the alignments for recombination events via the Datamonkey web server (www.datamonkey.org). The General Reversible (REV) model was chosen for GARD based upon the Datamonkey model selection tool. In addition, for the GARD analysis, the site to site rate variation of beta-gamma was chosen with 6 rate classes. The Datamonkey web server was also used to perform the Branch-site random effects likelihood (Branch-site REL) method to compute average *dN*/*dS* ratios for each branch of the phylogeny using sequence alignments for all OXPHOS and nuclear genes (Kosakovsky Pond et al., 2011). The pruned Betancur-R et al. (2013) phylogeny was appended to each sequence alignment in HyPhy and saved as one nexus file. Branch lengths were not included. This was then uploaded to the Datamonkey web server and the "user tree" option was used. Branch-site REL was chosen because it allows substitution rates to vary across all branches simultaneously. These computations using Branch-site REL were only performed to visualize the patterns of *dN*/*dS* in Figs. 1, 2, 3, and 4 as the hypothesis testing software did not output these values. Statistical results in this study are solely from the hypothesis testing software (RELAX), explained below.

The beta version of the hypothesis testing software package RELAX (obtained from the authors by personal communication prior to its formal release), is designed for detecting relaxed purifying selection between two groups (Wertheim et al., 2014); and this was used to compare *dN*/*dS* between the high performance and sedentary fishes. In describing RELAX, Wertheim et al. (2014) denote *dN*/*dS* as ω, and explain: "Our test for relaxed selection is based on the different effects relaxation has on ω values smaller than 1, representing purifying selection, and on ω values larger than 1, representing positive selection. When selection is relaxed, the smaller ω values increase towards 1, whereas ω values above 1 decrease". In this study, the sequence alignments for all 13 OXPHOS genes were loaded into HyPhy, and the RELAX.bf batch file (available from the Datamonkey web server and Wertheim et al. (2014)) was used to compare the high performance and sedentary groups as marked on the pruned phylogeny from Betancur-R et al. (2013) (Fig. 5). Branch lengths from Betancur-R et al. (2013) were not included in the RELAX analysis. Node support values are provided as supplementary information. A maximum likelihood phylogeny was also computed from the concatenated alignment of all 13 OXPHOS genes to examine the influence of phylogeny choice on the RELAX analysis (Supplementary information). These RELAX tests were run one short gene (ATP8, 165 bp) and two long genes (COX1, 1572 bp; CYTB, 1140 bp). Branch lengths were used for these tests. The vertebrate mitochondrial translation code was selected for the RELAX analysis. The same methodology was used for all three nuclear housekeeping genes by using the universal translation code.

In order to examine if positive selection also differs between these fishes, a mixed effects model of evolution (MEME) was chosen (Murrell et al., 2012). Separate phylogenies from Betancur-R et al. (2013) were created for the high performance and sedentary groups, and the main sequence alignment was also parsed accordingly. This was done because MEME outputs result for individual codons across entire alignments, making it difficult to identify differences between taxonomic groups. MEME was run on the Datamonkey web server using the general time reversible model (REV) substitution model and a significance level (p-value) of 0.05.

2.4. PIC

In order to examine the impact of inappropriately using PIC on *dN*/*dS* ratios as per Shen et al. (2009), PIC for mean *dN*/*dS* ratios were computed for concatenated sequences of all 13 OXPHOS genes in the Ape package for R (Paradis et al., 2004). Only PICs for *dN*/*dS* of branch tips were used. A two-sample *t*-test assuming unequal variances was used to compare the PIC results between the two fish groups.

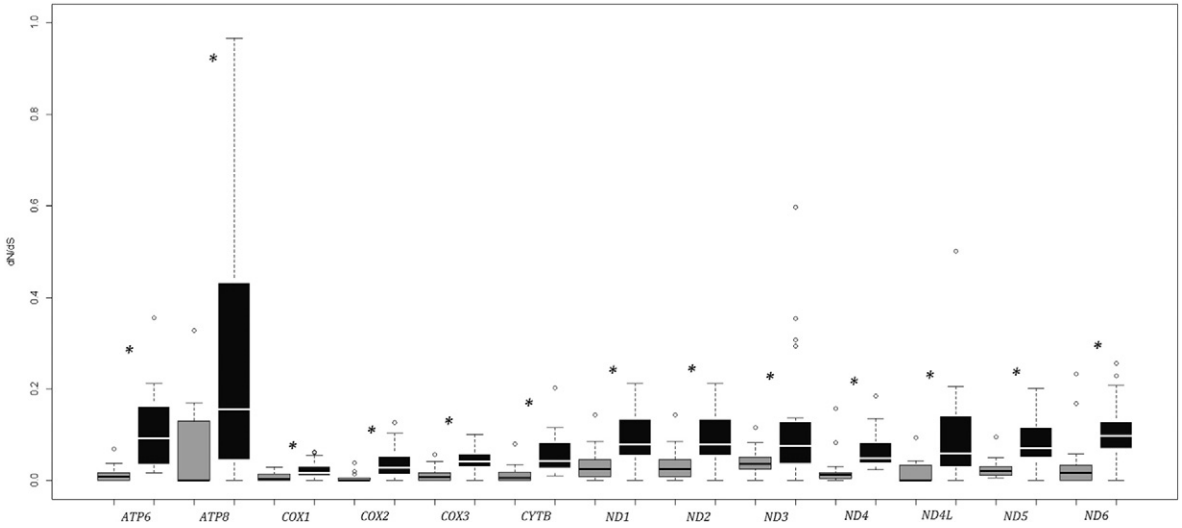

Fig. 1. Mean *dN*/*dS* ratios computed using Branch-site REL in the HyPhy package. Gray boxes depict *dN*/*dS* for high performance fish and black boxes represent sedentary fish. Hypothesis testing was performed using RELAX and asterisks indicate significant differences ($p < 0.05$). Some outliers are omitted here to make an efficient use of space, but no deletions were made for hypothesis testing.

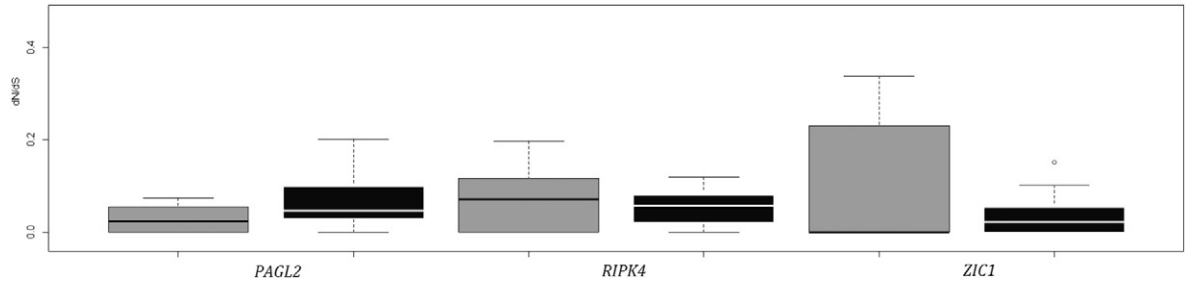

Fig. 2. Mean *dN*/*dS* computed in Branch-site REL for three housekeeping genes encoded by the nuclear genome that are not directly associated with OXPHOS. Results from RELAX indicate that *dN*/*dS* does not differ significantly ($p > 0.05$) between high performance and sedentary fish for these three genes. Gray boxes depict high performance fish, while black represents sedentary fish.

2.5. COX subunit binding site evolution

The nucleotide sequences for cytochrome c oxidase subunits I and III (*COXI*, *COXIII*) were aligned in MEGA6 for the same 48 species examined for the mitochondrial genome analysis. The locations of subunit binding sites were identified in the NCBI protein graphical sequence viewer 3.1 available in Genbank (http://www.ncbi.nlm.nih.gov/protein/). These locations were then marked in the multiple sequence alignments for *COXI* and *COXIII*. These main alignments were each subset into three alignments containing binding sites that 1) interact with binding sites of the mitogenome or 2) interact with binding sites of the nuclear genome, while the other contained 3) the protein background (non-binding sites) of *COXI*, and *COXIII*. The *dN*/*dS* values were computed for each alignment subset of each locus using the same methods employed in Branch-site REL as for the analysis of all 13 OXPHOS genes (described above). Weighted mean was used in Branch-site REL as it performed better with the short length of these sequences ($\omega 1$ * prob ($\omega = \omega 1$) + $\omega 2$ * prob ($\omega = \omega 2$) where $\omega = dN/dS$ in each of the three rate classes computed). The beta version of RELAX was again used for all hypothesis testing.

3. Results

In the mitogenome dataset, *Ts*/*Tv* shows a bias towards transitions with an average ratio of 1.86 ± 0.07 (Table 1). All 13 mitochondrial OXPHOS genes of sedentary fish show significantly higher (via RELAX) *dN*/*dS* ratios (i.e. relaxed purifying selection) relative to high performance fish (Fig. 1) (Raw values for each species and gene are available in Supplementary Dataset 2). RELAX test results that were obtained using the mitogenome phylogeny computed in this study, did not lead to any changes in significant differences when compared to the original analysis ($p < 0.05$) (Supplementary information). MEME identified twice as many sites (31 versus 16) that may be under positive selection in the sedentary group when compared with the high performance group (Table 1). None of these sites, under positive selection, were shared between groups. Exact positions are available in Supplementary Dataset 1. Notably, the three nuclear housekeeping genes showed no significant differences in *dN*/*dS* ratios between either groups (Fig. 2). Also, the *COXI* and *COXIII* subunit binding sites, that interact with subunits encoded by either the nuclear or mitogenomes, showed no significant differences in *dN*/*dS* ratios within or between both groups of fish. However, we did detect significant differences between the protein backgrounds of *COXI* and *COXIII*, i.e. domains that do not contain binding sites, between both groups of fishes. P-values for all RELAX likelihood ratio tests are available in Supplementary List 5. Lastly, when revisiting the impact of incorrectly applying PIC to *dN*/*dS* computed from concatenated alignments of all 13 genes, as per Shen et al. (2009), we observe that their method (via *t*-Test: two-sample assuming unequal variances) obscures significant differences between high performance and sedentary fishes, revealed when using independent contrasts via RELAX ($p = 0.29$ versus $p < 0.0001$, respectively) (Fig. 4).

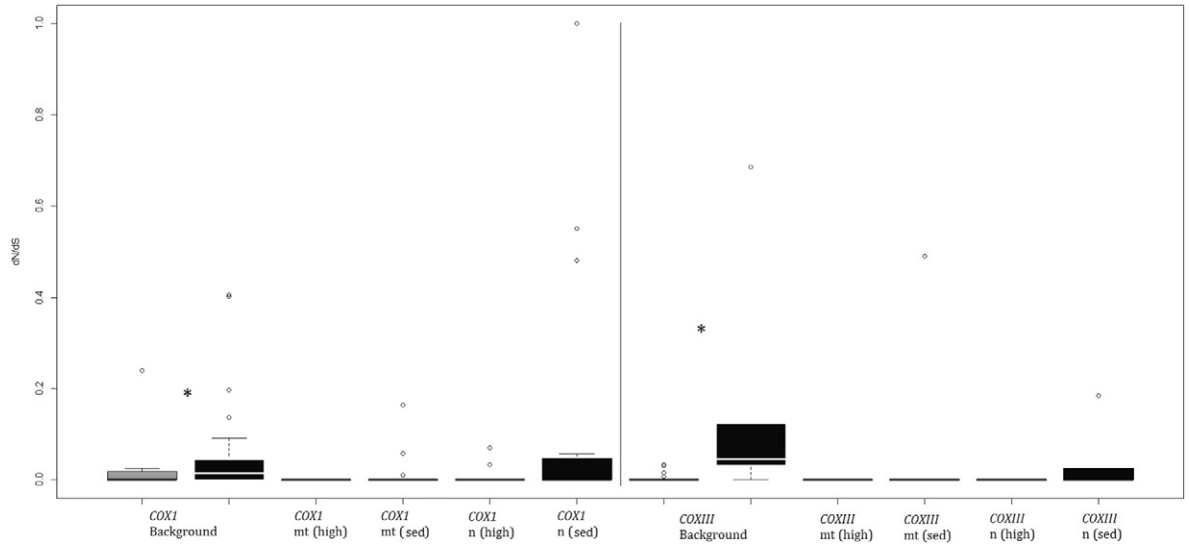

Fig. 3. Weighted mean *dN*/*dS* ratios computed using Branch-site REL for *COXI* and *COXIII* subunit binding sites that bind subunits encoded by the nuclear or mitochondrial genomes. *dN*/*dS* for the protein background (all residues not part of a binding site) was also computed. Black represents sedentary fish (sed) and gray represents high performance (high). Binding sites that interact with subunits encoded by the nuclear genome are signified with 'n'. Binding sites that interact with subunits encoded by the mitochondrial genome are signified with 'mt'. All hypothesis testing was done using RELAX. Asterisks indicate significant differences ($p < 0.05$).

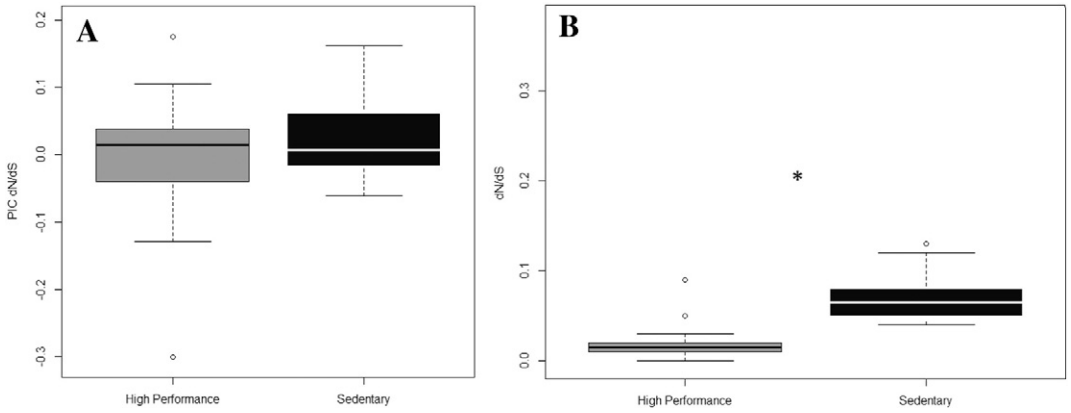

Fig. 4. A) Inappropriate use of phylogenetically independent contrasts (PIC) on *dN*/*dS* values as per Shen et al. (2009) results in the removal of the significant differences ($p = 0.29$). These *dN*/*dS* ratios were computed using Branch-site REL for the concatenated sequences of all 13 OXPHOS genes. B) *dN*/*dS* values without the application of PIC. These are significantly different via RELAX ($p < 0.05$). It is not possible for A) and B) to share the same y-axis due to the PIC transformation of *dN*/*dS*.

4. Discussion

Sedentary fishes appear to possess elevated *dN*/*dS* ratios for all 13 OXPHOS genes of their mitogenomes when compared with high performance fishes (Fig. 1); and the fact that this pattern does not exist for the three nuclear housekeeping genes we examined (Fig. 2), suggests that this could be due to evolutionary processes acting uniquely on mitochondrial DNA. It should be noted that purifying selection dominates the mitogenomes of both fish groups and only a few outliers across all 13 genes possessed *dN*/*dS* ratios outside the range of 0 to 0.25 (Fig. 1). Some sites were identified as being under positive selection, and with twice as many being identified in the sedentary group (Table 1). These sites are spread across multiple OXPHOS genes and are not consistent between the two groups. An in-depth analysis of the function implications of selection at these sites is beyond the scope of this study, but they do provide an independent line of evidence that corroborates the notion that sedentary fish accumulate more mutations.

We hypothesize that a relaxation of purifying selection may account for the significantly elevated *dN*/*dS* ratios observed in the sedentary group. This may explain why high performance fishes such as tuna and billfishes are difficult to identify from congeners using mitochondrial genetic-distance based approaches (Lowenstein et al., 2009; Hanner et al., 2011). However, we recognize experimental manipulations to test that our observations will be difficult to perform, because these fish have complex life histories, and lengthy generation times. Also, discussion of adaptive selection should involve its location and timing (Mayr, 1983; Vitti et al., 2013); which in this case requires information about germ-line mitochondrial transmission, and differential offspring survival that will also be difficult to obtain. Despite these limitations, we have found similar patterns, hypotheses, and related experimental approaches throughout the mitochondrial literature, and we present a comparative view of these throughout the discussion.

While Shen et al. (2009) did arrive at the same conclusions as this study, i.e. that taxa with greater energy requirements experience greater mitogenome purifying selection, some of their statistical methods are incorrect. In particular, they transformed the *dN*/*dS* values that they computed using PIC (Felsenstein, 1985); doing this is not appropriate for calculating branch specific *dN*/*dS* values because the values are

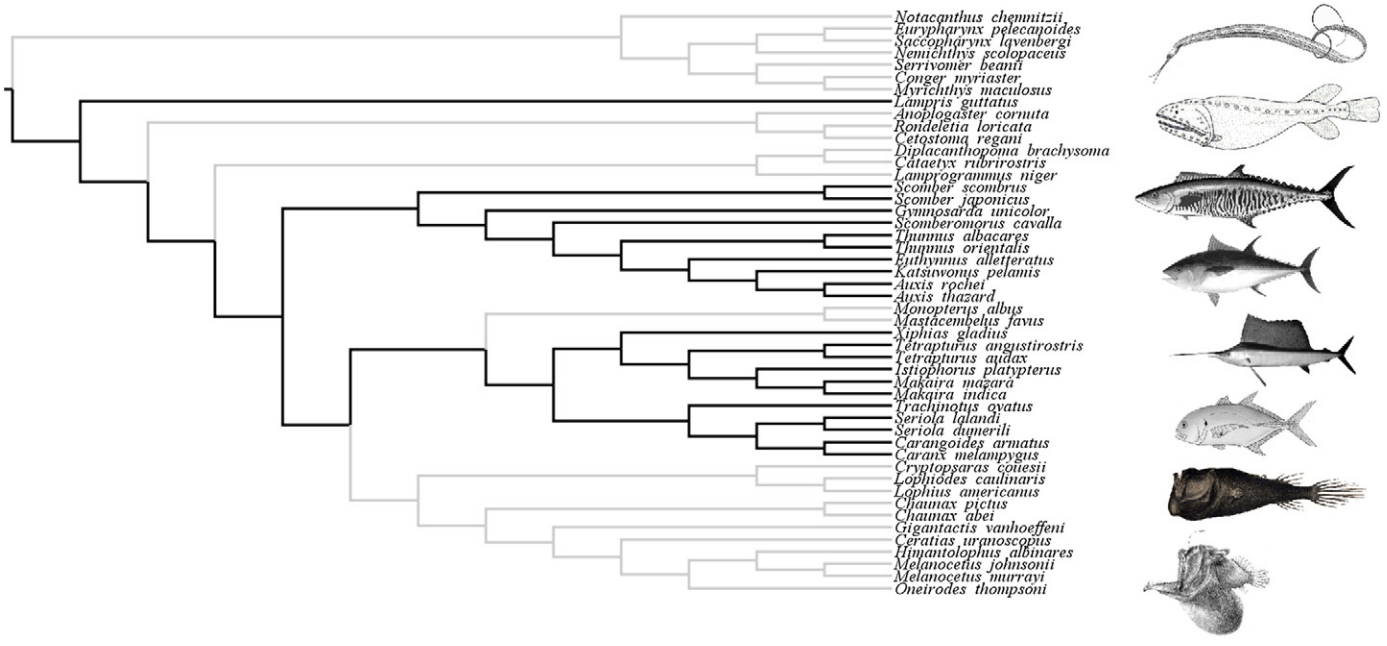

Fig. 5. Pruned topology of the maximum likelihood phylogeny generated by Betancur-R et al. (2013) used in this study. Tips with black branches were marked in RELAX as the high performance group while tips in gray were marked as the sedentary group. Images representing members of these groups were obtained from the Wikimedia Images Commons.

Table 1
Results from MEME tests for diversifying selection on separate concatenated sequence alignments from the high performance and sedentary groups. Number of codons per gene that were identified as being under selection are shown. Exact positions are available in Supplementary Dataset 1. The ratios of transitions to transversions (R) computed from the entire dataset are also shown.

	# Codons with positive selection detected			
Gene	High performance	Sedentary	R = *Ts/Tv*	Standard error
ATP6	1	1	1.63	0.06
ATP8	2	5	1.94	0.14
COX1	2	1	2	0.04
COX2	1	1	2.25	0.09
COX3	0	1	1.86	0.07
CYTB	0	0	1.68	0.04
ND1	0	1	1.72	0.05
ND2	2	3	1.47	0.03
ND3	0	0	1.69	0.09
ND4	0	4	1.78	0.04
ND4L	1	2	1.86	0.1
ND5	3	5	1.85	0.04
ND6	4	7	2.42	0.09
Total	16	31		

already a contrast whenever they are computed by comparing two nodes on a branch (see Popadin et al. (2007) for this, and other reasons why PIC is inadmissible in this case). When examining the impact of such an error, we determined that PIC actually removes significant differences between the two groups of fish (Fig. 4 A,B), and Shen et al.'s (2009) ability to detect significant differences may also be related to the fact that they only computed PIC from a small sample of their taxa, for reasons that were not explained.

The literature departing from the original paradigm of neutral mitogenome evolution has been steadily growing (reviewed by Meiklejohn et al. (2007)), especially in the light of dispersal, effective population size (N_e), and metabolic rate. An early example comes from a study examining the evolution of human mitogenomes, which rejected the neutral evolution model for some human populations based on restriction fragment length polymorphism (RFLP) data by providing evidence that a distance based approach may be insufficient to describe population divergence events (Excoffier, 1990). A more recent study, examining insects, found that flying lineages possess lower *dN/dS* ratios compared to flightless lineages (Mitterboeck and Adamowicz, 2013), and similar to our hypothesizing, the authors suggest that these patterns may be due to increased selection on OXPHOS energy generation for flighted lineages. Also similar to our results, Mitterboeck and Adamowicz (2013) found that nuclear gene *dN/dS* ratios do not mimic those of mitochondria, and they further suggest that decreased dispersal following loss of flight may reduce effective population sizes (N_e), and increase a lineage's susceptibility to deleterious mutations due to increase in genetic drift. This suggestion is supported by a recent study of mammalian nuclear genes (Popadin et al., 2013), finding that species with small N_e show relaxation of purifying selection; and in general purifying selection is 5-fold greater for mitogenomes compared to nuclear genomes. In another recent study, it was determined that mitogenomes are under significantly more intense purifying selection when compared with nuclear genes of the OXHPOS pathway (Nabholz et al., 2013). And to support this, Nabholz et al. (2013) cite a growing body of literature that proposes a negative correlation between gene expression and evolutionary rate, based on observations of significantly lower gene expression in nuclear OXPHOS genes when compared to the mitogenome.

Differences in the standard metabolic rates (metabolic rate at rest) of high performance versus sedentary fishes may also contribute to our observed differences in *dN/dS*. High performance fishes, especially the tunas, have been documented to have some of the highest standard metabolic rates of any fish (Bushnell and Jones, 1994; Brill, 1996; Sepulveda and Dickson, 2000). Conversely, sedentary fishes such as eels and anglerfishes occupy the low end of the metabolic rate spectrum (Walsh et al, 1983; Cowles and Childress, 1995; Bishop and Torres, 1999; Clarke and Johnston, 1999; McKenzie et al., 2000). This phenomenon has also been observed in insects whereby selection seems to support higher standard metabolic rates in flying compared to sedentary taxa (Reinhold, 1999). Despite the appeal of these broad patterns, experimentally determining a causal link between *dN/dS* and standard metabolic rate appears to be difficult, and the relevant literature seems sparse. However, one recent study examined metabolic flux in three enzyme systems of human erythrocytes (flux is based on the production rate of metabolites), and found that enzymes with high fluxes were under the greatest purifying selection (Colombo et al., 2013). Mitogenomes of salamanders, which have the lowest energy needs among tetrapods, show relaxed purifying selection when compared with frogs (Chong and Mueller, 2013); although there is considerable debate about the influence of metabolic rate on molecular evolution, given complications presented by body size and temperature (reviewed by Glazier (2014)). The primary mechanism by which metabolic rate is conventionally thought to influence the evolution of mitochondrial DNA, is DNA-damage-and-repair from an OXPHOS by-product – known as reactive oxygen species (ROS) – such as oxygen ions, and peroxides. A review by Galtier et al. (2009) rejects the ROS hypothesis, on grounds that the metabolic rate of female germ-line cells, in general, may not reflect that of musculature; and generally argues that the relationships between metabolic rate, ROS, and mutation rate are complex, and confounded by many factors. Galtier et al. (2009) also point to a comprehensive study of >300 metazoans (Lanfear et al., 2007), which failed to find the link between mass-specific metabolic rate and molecular evolution which was proposed by Gillooly et al. (2007).

In this study, the ROS hypothesis alone doesn't explain the difference in *dN/dS* between the sedentary and high performance fishes, because our results are opposite the ROS expectation, with *dN/dS* being significantly lower in taxa with high standard metabolic rates. While *dN/dS* does not necessarily reflect the rate at which these taxa accumulate mutations, given that these ratios are so low (Fig. 1), it is possible that the rate is also low. Of relevance to fishes, Betcanur-R et al. (2013) performed a fossil calibrated molecular clock analysis, and estimated the lineage age for most species in this study, so estimates of substitution rates are possible but beyond the scope of our work here. Lastly, given our results, it may also be reasonable to hypothesize that purifying selection and standard metabolic rates are correlated in high performance fishes, because purifying selection is necessary due to their high energy requirements.

A relaxation of purifying selection was not detected when comparing the subunit binding sites in *COXI* and *COXIII* for high performance and sedentary fishes, regardless of their interaction with subunits encoded by the mitogenome or the nuclear genome. However, the *COXI* and *COXIII* subunits that interact with the nuclear genomes did have higher *dN/dS* values in sedentary fishes (Fig. 3), but these differences were not significant. The fact that the *dN/dS* of the protein backgrounds (not binding sites) of *COXI* and *COXIII* were significantly different does not come as a surprise, as this was also true for the entire genes (Fig. 1). This suggests that protein domains other than binding sites are accumulating the additional mutations in sedentary fishes. The fact that relaxed purifying selection was not detected in subunit binding sites suggests that they may be of such structural or functional importance, that mutations in these regions are highly deleterious, even for sedentary sit-and-wait predators with low energy requirements. Recently, Zhang and Broughton (2013) cast some doubt on the ubiquity of mito-nuclear coevolution in vertebrates by examining 13 mitochondrial OXPHOS genes, 60 nuclear OXPHOS genes and 77 non-OXPHOS nuclear genes. They suggest that mito-nuclear coevolution has a small contribution to the evolution of the OXPHOS pathway, and the accelerated evolution detected in nuclear subunits in other studies may in fact be due to a relaxation in functional constraint on non-core subunits. Also, as mentioned in our introduction, cybrid crossing experiments have

clearly demonstrated that mito-nuclear incompatibilities from different haplotypes exist, and result in fitness losses (McKenzie et al., 2003; Niehuis et al., 2008; Arnqvist et al., 2010). Given this conflicting evidence, it seems that more work is required to integrate our knowledge between genomic and organismal scales. The overarching message here may be that mito-nuclear coevolution does occur, but the substitutions driving it in vertebrates appear rare.

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Conflicts of interest

None are declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2015.06.074.

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