Heterogeneous natural selection on oxidative phosphorylation genes among fishes with extreme high and low aerobic performance

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Abstract

Background: Oxidative phosphorylation (OXPHOS) is the primary source of ATP in eukaryotes and serves as a mechanistic link between variation in genotypes and energetic phenotypes. While several physiological and anatomical factors may lead to increased aerobic capacity, variation in OXPHOS proteins may influence OXPHOS efficiency and facilitate adaptation in organisms with varied energy demands. Although there is evidence that natural selection acts on OXPHOS genes, the focus has been on detection of directional (positive) selection on specific phylogenetic branches where traits that increase energetic demands appear to have evolved. We examined patterns of selection in a broader evolutionary context, i.e., on multiple lineages of fishes with extreme high and low aerobic performance.

Results: We found that patterns of natural selection on mitochondrial OXPHOS genes are complex among fishes with different swimming performance. Positive selection is not consistently associated with high performance taxa and appears to be strongest on lineages containing low performance taxa. In contrast, within high performance lineages, purifying (negative) selection appears to predominate.

Conclusions: We provide evidence that selection on OXPHOS varies in both form and intensity within and among lineages through evolutionary time. These results provide evidence for fluctuating selection on OXPHOS associated with divergence in aerobic performance. However, in contrast to previous studies, positive selection was strongest on low performance taxa suggesting that adaptation of OXPHOS involves many factors beyond enhancing ATP production in high performance taxa. The broader pattern indicates a complex interplay between organismal adaptations, ATP demand, and OXPHOS function.

Background

Physiological processes may serve as mechanistic links between genotypes and organismal phenotypes. Accordingly, adaptations in genes of energy metabolism pathways may facilitate the evolution of organismal structures and life habits with diverse energy requirements. Proteins encoded by the mitochondrial genome serve as core subunits of the oxidative phosphorylation (OXPHOS) system, the primary source of ATP in eukaryotic cells. Consequently, organismal traits with differing ATP demands may be influenced by adaptation in mitochondrial genomes [1–5]. Our understanding of patterns and rates of adaptive change in mitochondrial genomes remains limited despite extensive use of mitochondrial genes as molecular markers in evolutionary studies [6] and the important role of mitochondria in many human pathologies [7]. Although there is ample evidence of natural selection acting on mitochondrial genes [8–15], the functional significance of adaptive mitochondrial change is rarely known.

Evidence of positive selection on OXPHOS genes has been associated with evolution of a variety of energetically demanding characteristics (reviewed in [16]), including origin of large brains in anthropoid primates [17], powered flight in bats [18], and adaptation to cold environment in polar bears [19]. These suggest a significant role for OXPHOS in organismal adaptation, and because divergence among lineages often involves traits with
different energy usage, OXPHOS evolution may be an important factor in the diversification of life. OXPHOS functional efficiency may be particularly important to energy intensive processes such as locomotion. Variation in locomotive performance of fishes is among the most extreme among vertebrates, ranging from largely sedentary filter feeders and sit-and-wait predators to highly migratory species and active pelagic foragers. For example, seahorses and flounders spend much of their time nearly motionless, while tunas and marlins are "high-performance" swimmers that exhibit high aerobic metabolism and prolonged fast swimming [20, 21]. Highly-active fish taxa exhibit many morphological and physiological adaptations that enhance swimming performance (reviewed in [22]). Such adaptations include modifications of body shape and hydrodynamics [23–25], swimming form and mechanical kinematics [26–28], muscle composition [29–31], metabolic rates [32], heat volume and aerobic capacity [32–34], and mitochondrial structure and concentration [35]. However, much less is known about the molecular adaptations that influence organismal performance.

At the molecular level, expression levels of proteins directly involved in energy metabolism may be increased in highly mobile fish species. Tunas and marlins, which have higher cruising speeds than other active fishes [21], have been shown to have elevated myoglobin [36] and higher concentrations of metabolic enzymes in heart and skeletal muscle (reviewed in [36]). Elevated activities of citrate synthase (which catalyzes the first reaction of the Krebs cycle), carnitine-palmitoyl transferase, and 3-hydroxy-o-acyl-CoA dehydrogenase (rate-limiting enzymes in fatty acid oxidation), reflect the increased aerobic metabolic potential of scombrid fishes [35]. In addition, OXPHOS genes are differentially expressed between morphs of lake whitefish that differ in activity levels in foraging behavior [37].

Alternatively, or in addition, to variation in gene expression and post-transcriptional modification, divergent energy demands may lead to adaptive evolution in the structure of specific OXPHOS proteins. Variation in OXPHOS proteins could influence the efficiency of ATP production by affecting how tightly electron transport and proton pumping are coupled in the electron transport chain. Modifications in the structure of OXPHOS complexes I–IV caused by amino acid substitutions in constituent proteins could affect "slip reactions", resulting in more or less protons pumped by the electron-transport-chain for each electron pair transferred (the $\text{H}^+/2e^-$ ratio) (reviewed in [38]). Alternatively, substitutions in proteins of ATP synthase could modify the amount of ATP made for each proton driven through it (the $\text{H}^+/\text{ATP}$ ratio) [38]. These phenomena are consistent with prior reports of positive selection in the cytochrome c oxidase genes of the high performance billfishes [39, 40], the $\text{MT-ND2}$ and $\text{MT-ND5}$ genes of some migratory Pacific salmon [41], and $\text{MT-ND2}$, $\text{MT-ND4}$ and $\text{MT-ND5}$ genes of pelagic Atlantic herring [42].

Here, we investigate patterns of adaptation on OXPHOS genes in diverse group of fish taxa with different swimming performance. We hypothesized that positive natural selection would affect OXPHOS efficiency among divergent lineages with long-term differences in ATP demand. Thus molecular adaptation was predicted to be associated with locomotor intensity (speed, duration and frequency). We examined evidence for positive selection on all mitochondrial OXPHOS genes from six fish groups that can be classified into three different swimming performance categories based on general locomotion patterns [22]. Tunas and billfishes represent high performance swimmers, mackerels and jacks represent moderate (or moderate-high) performance swimmers, and flatfishes and seahorses + pipefishes represent low performance swimmers. Pelagic fishes exhibit highly aerobic locomotion with greater endurance than sedentary fishes, and among the pelagic fishes, tunas and billfishes may maintain the highest speeds for the longest duration [22]. Seahorses and pipefishes exhibit much lower frequency, duration, and speed of locomotion, while flatfishes exhibit lower frequency, and sustained speed. These factors as well as their unusual swimming forms and kinematics suggest that much less of their total energy budget is devoted to locomotion, so we categorized them as low-performance swimmers. Recent phylogenetic analyses [43–45] indicate that these taxa are arranged into two monophyletic groups each containing representatives of all three performance classes (Fig. 1). Two previous studies [39, 40] examined positive selection in some high-performance fishes that are included in this study. We extended these studies by including all 13 mitochondrial OXPHOS genes and examined the evidence of positive selection on multiple phylogenetic branches, including six independent fish lineages as well as some ancestral branches. In contrast to previous studies, this approach allowed us to make mitochondrial genome-wide assessments of selection across taxa exhibiting a wide range of locomotor performance. We also inferred functional significance from the position of positively selected amino acid sites in the 3-dimensional structure of specific enzyme complexes. Our results indicate that selection on OXPHOS genes is indeed associated with divergent swimming habits among these fishes. However, selection is heterogeneous over evolutionary time and positive selection is not strictly associated with high performance taxa. Thus we provide new insights on the evolution of swimming diversity in fishes and the adaptation of OXPHOS genes relative to organismal energetic performance on a broad phylogenetic scale.
Methods

Phylogeny reconstruction

We acquired sequences of 13 mitochondrial protein genes (all of them encode subunits in OXPHOS) from the representative species of each fish group from National Center for Biotechnology Information (NCBI) GenBank (Additional file 1: Table S1). To complement the mitochondrial genes, we added 7 nuclear genes which are commonly used in fish phylogenies (obtained from NCBI GenBank) or were developed as part of the Fish Tree of Life project [46]. These genes are \textit{rag1} (recombination activating protein 1), \textit{rag2} (recombination activating protein 2), \textit{rhodopsin}, \textit{tmo4c4} (anonymous, see [47]), \textit{zic1} (zinc finger protein 1), \textit{myh6} (myosin, heavy chain six), and \textit{btbd7} (BTB domain containing seven). For species without available sequences, we designed primers (Additional file 1: Table S2) and amplified and sequenced specific genes via standard polymerase chain reaction (PCR). PCR product was sequenced in both forward and reverse directions using ABI BigDye terminator chemistry and an ABI Prism 3130 XL Genetic Analyzer. Sequences have been deposited in GenBank (Additional file 1: Table S3). Particular genes for a few species could not be amplified. In such cases, sequences were obtained from congener, yielding 19 “chimaeric” individuals. The 20 genes (13 mitochondrial and 7 nuclear) were concatenated for phylogenetic analyses [48].

Maximum likelihood trees (with 1000 bootstrap replicates) were estimated in the program RAxML v.7.0.4 [49]. Bayesian phylogenetic analysis was performed with MrBayes v.3.1 [50, 51]. Swimming performance states were reconstructed from extant taxa with parsimony and likelihood using outgroups from [44] in Mesquite [52].

Analysis of patterns of natural selection

Sequences of 13 mitochondrial OXPHOS genes were used to examine patterns of natural selection. We first
ran a series of random sites models (M0, M1a, M2a, M3, M7, M8a and M8) implemented in Codeml of PAML v.4.7 [53]. Likelihood ratio tests (LRTs) were conducted on the likelihood values produced by specific pairs of models: M3 vs. M0, M2a vs. M1a, M8 vs. M7, and M8 vs. M8a. Next we ran branch-site models on designated branches with three different starting ω values (0.1, 1.0, and 4.3). We had no prior knowledge of which branch(es) could have experienced positive natural selection (except for branches leading to tunas and billfishes); therefore, we designated one branch (from b1-25 in Fig. 2) as the foreground branch in each test. LRTs were performed to determine if the more complex model A is significantly better than the null model. In the branches that showed evidence of positive selection, we used Bayes empirical Bayes (BEB) to calculate the probability of amino acid sites are under positive selection.

The 13 mitochondrial gene sequences were also analyzed using TreeSAAP [54], which measures the selective influences on 31 structural and biochemical amino acid properties, and performs goodness-of-fit and categorical statistical tests. We used a sliding window size of 20 amino acids (Additional file 1: Figure S2). Because of the large number of sites under positive selection, we only show sites with significance when its p < 0.001 for clarity. Then we used a sliding window size of 1 and one amino acid site is defined as being positively selected as long as one of the 31 properties showed significance.

Mapping positively selected amino acid sites onto 3-dimensional (3D) crystal protein structure

The MT-ND genes that encode subunits in OXPHOS complex I are considered highly conserved between prokaryotes and vertebrates [55]. We inferred the potential function of positively selected amino acid sites belonging to MT-ND subunits by mapping them onto the 3D crystal structure of Thermus thermophiles (PDB ID: 4HEA), a Gram negative eubacterium [56]. We mapped the positively selected sites of MT-CYB onto chicken bc1 complex C chain (PDB ID: 1BCC) crystal structure because a study suggests the structure of the catfish MT-CYB protein resembles that of chickens [57]. Similarly, we mapped the positively selected sites onto bovine MT-COI-3 subunits 3D structure (PDB ID: 1OCC) because of the highly conserved structure from bacteria to bovine [58, 59]. All the mapping was conducted using Genious pro (v.7.0).

Results

Phylogeny and ancestral state reconstruction

Both maximum likelihood and Bayesian analyses generated the same topology (Fig. 2) regardless of partition scheme with relatively high bootstrap support and posterior probabilities (see Additional file 1: Figure S1). The recent comprehensive phylogenetic analysis of [44] resolved the phylogeny of bony fishes to the level of taxonomic family, while a study by [45] focused on relationships of several pelagic fish groups. Our results are consistent with both of these studies for the taxa in common to each. Specifically, we recovered two monophyletic groups, each containing taxa with low, moderate, and high swimming performance. In one group, the tunas and mackerels (Scombriformes) are sister to the seahorses and pipefishes (Syngnathiformes), while the series Carangaria contains the billfishes (Istiophoriformes), jacks (Carangiformes), and flatfishes (Pleuronectiformes) [44] (see also Phylogenetic Classification of Bony Fishes—Version 3: www.deepfin.org).

Swimming performance states were reconstructed from extant taxa with parsimony and likelihood using outgroups from [44]. These results of reconstructed swimming performance suggest that the ancestors of each major group were low performance swimmers (Fig. 1), although incomplete taxon sampling of related groups could mislead the reconstructions. Even if the ancestors were moderate performance swimmers, it is clear that high performance swimming evolved independently in the tunas and billfishes.

Analysis of positive selection

Positive selection under site models cannot identify particular branches where positive selection has occurred, but they can detect positive selection among sites where it occurs as long-term trends or among multiple branches separated on the tree. A significant difference was detected between model M0 and M3, which suggests ω is variable among sites (Table 1). We used three model pairs to test for positive selection: M1a vs. M2a, M7 vs. M8, and M8a vs. M8. Neither M2a-M1a nor M8a-M8 showed significant difference. However, M8-M7 showed a significant difference, suggesting some positive selection signal on certain sites somewhere on the tree.

Table 2 lists the resulting likelihood values, likelihood ratio tests (LRTs), and estimated model parameters for each branch examined under branch-site models. We performed a false discovery rate analysis [60] on these results where we performed 20 tests and recovered 12 positive results. All 12 positive results had a q value of 0.03 or less, and the probability of a false positive among them is 0.36. Because this is less than 1.0, no false positives are expected. The number of amino acid sites on each branch inferred to be under positive selection via Bayes Empirical Bayes is provided Additional file 1: Table S4 (the identity of these sites and more details are provided in Additional file 1: Table S5). These results showed substantial differences in natural selection on lineages leading to taxa with different swimming performance. We found significant evidence of positive selection on the ancestral lineages of all three performance categories (branches b2, b17, b3,
Fig. 2 (See legend on next page.)
and b4 in Fig. 2), lineages leading to moderate-performance swimmers (b6 and b13), and low-performance swimmers (b16, b23, b24, b25, and b11). Conversely, strong purifying (negative) selection was identified on lineages of high-performance swimmers (b7, b8, b9, b10, b14, and b18). Fourteen sites that appeared to be positively selected occur on multiple branches; however, they are not associated with branches leading to a particular performance group (see details in Additional file 1: Table S5).

The above tests assess the strength of the evidence for positive selection rather than the strength of selection itself. An indicator of the strength of positive selection on a branch can be obtained from the product of ω = 1 and the estimated ω value for those sites. In Fig. 2, the branch width is shown proportional to the strength of positive selection based on this measure (except for b12, b23 and b24 which had extremely high ω and a cap of ω = 50 is used). Among those branches harboring sites under positive selection, branches ancestral to more than one swimming category (b2, b3, b4, b12, b17) and branches leading to low-performance fishes (b11 and b23) exhibited greater selection than branches leading to moderate-performance fishes (b6 and b13), and substantially more than any branches associated with high-performance fishes. This is counter to the notion that high performance swimmers will have been most strongly influenced by positive selection for enhanced OXPHOS performance assuming enhanced OXPHOS efficiency is the major contributor to high performance. However, there are alternative contributors to high performance such as increased mitochondrial density per tissue mass, more closely packed inner mitochondrial membrane cristae, increased metabolic enzyme activity, increased expression of genes involved in a number of biological pathways such as glycolysis, protein biosynthesis, and cytoskeletal structure. Under any of the later scenarios, an increase in OXPHOS efficiency might not be necessary for high performance.

The physico-chemical properties analysis as implemented by TreeSAAP [54] does not provide inferences about particular branches on the phylogeny, but does provide information about changes of particular amino acid sites across the whole tree. A large number of amino acid sites were identified as having substitutions with significant physico-chemical differences; presented in Additional file 1: Figure S2 and Table S4. A mitochondrial genome-wide (only the protein coding genes) sliding window analysis (window size = 20 amino acids) revealed that the vast majority of windows contained substitutions that exhibited between 0 and 15 properties with significant differences (Additional file 1: Figure S2). Windows with substitutions exhibiting between 15 and 30 significant properties occurred at much lower frequency. Although windows with the greatest number of significant property differences were observed in the genes for MT-ND1, MT-CO1, MT-ND4 and MT-CYB, there were no other apparent patterns of variation in the distribution of such sites across the genome (Additional file 1: Figure S2). Positively-selected sites identified by both methods are summarized in Additional file 1: Table S4, S5.

### Table 1 Results of PAML Random Sites models

| Model | lnL | Parameters | Null LRTs | p |
|-------|-----|------------|-----------|---|
|       |     | ω0/0 p  | ω0/ωq  | ω0/ωq  | p |
| M0    | -191327.6225 | 0.03421 |          |          |   |
| M1a   | -189631.0183 | 0.02838 (94.9 %) | 1 (5.1 %) |          |   |
| M2a   | -189631.0190 | 0.02838 (94.9 %) | 1 (5.1 %) |          |   |
| M3    | -184769.7796 | 0.00609 (72.6 %) | 0.11443 (27.4 %) | 79.11600 (0) | M1a 0 |
| M7    | -184829.5771 | 0.22705 | 4.62358 |          |   |
| M8a   | -184813.0237 | 0.23248 | 5.02542 | 1.00000 |   |
| M8    | -184813.0237 | 0.23248 | 5.02544 | 1.00000 | M7 33.1068 ** |

InL: log likelihood; LRTs represents the likelihood ratio tests; 2 * (lnL(Model) – lnL(Null)).** represents p < 0.01

ω values of each site class are shown for models M0-M3 (ω0 – ωq) with the proportion of each site class in parentheses. For M7-M8, the shape parameters, p and q, which describe the beta distribution are listed.
| Branch | Model A  | Null model | LRTs | Site class | 0   | 1   | 2a | 2b |
|--------|---------|------------|------|------------|-----|-----|----|----|
| b1     | -189631.0183 | -189631.0183 | 0   | Proportion | 0.94985 | 0.05015 | 0.00000 | 0.00000 |
|        |          |            |      | Foreground ω | 0.02838 | 1.00000 | 1.00000 | 1.00000 |
| b2     | -189619.8114 | -189623.914  | 8.2052** | Proportion | 0.92062 | 0.04856 | 0.02927 | 0.00154 |
|        |          |            |      | Foreground ω | 0.02825 | 1.00000 | 18.30494 | 18.30494 |
| b3     | -189710.2567 | -189714.0692 | 7.6250** | Proportion | 0.92700 | 0.04823 | 0.02355 | 0.00123 |
|        |          |            |      | Foreground ω | 0.02811 | 1.00000 | 5.62025 | 5.62025 |
| b4     | -189591.2640 | -189598.5732 | 14.6183** | Proportion | 0.92799 | 1.00000 | 9.01795 | 9.01795 |
| b5     | -189728.0764 | -189729.5338 | 2.9148 | Proportion | 0.91976 | 0.04869 | 0.02997 | 0.00159 |
| b6     | -189719.7435 | -189722.2222 | 4.9573* | Proportion | 0.93956 | 0.04997 | 0.00994 | 0.00053 |
| b7     | -189738.4458 | -189738.4458 | 0   | Proportion | 0.94477 | 0.05030 | 0.00468 | 0.00025 |
| b8     | -189738.5873 | -189738.5873 | 0   | Proportion | 0.94946 | 0.05054 | 0.00000 | 0.00000 |
| b9     | -189738.5873 | -189738.5873 | 0   | Proportion | 0.94946 | 0.05054 | 0.00000 | 0.00000 |
| b10    | -189738.5873 | -189738.5873 | 0   | Proportion | 0.94946 | 0.05054 | 0.00000 | 0.00000 |
| b11    | -189613.4925 | -189622.0967 | 17.2084* | Proportion | 0.94688 | 0.04932 | 0.00362 | 0.00019 |
| b12    | -189615.9415 | -189619.0732 | 6.7338** | Proportion | 0.92922 | 0.04889 | 0.02080 | 0.00109 |
| b13    | -189610.5139 | -189616.7321 | 12.4364** | Proportion | 0.94411 | 0.04933 | 0.00624 | 0.00033 |
| b14    | -189734.9950 | -189736.0673 | 2.1446 | Proportion | 0.94724 | 0.05043 | 0.00221 | 0.00012 |
| b15    | -189624.9748 | -189628.0111 | 6.0725** | Proportion | 0.94529 | 0.04967 | 0.00479 | 0.00025 |
| b16    | -189621.7633 | -189627.8443 | 12.1622** | Proportion | 0.94863 | 0.04955 | 0.00173 | 0.00009 |
| b17    | -189738.5873 | -189738.5873 | 0   | Proportion | 0.94945 | 0.05055 | 0.00000 | 0.00000 |
| b18    | -189618.7575 | -189625.3146 | 13.1142** | Proportion | 0.93161 | 0.04861 | 0.01879 | 0.00098 |
| b19    | -189578.5777 | -189597.2838 | 37.4121** | Proportion | 0.90294 | 0.04769 | 0.04689 | 0.00248 |
| b20    | -189591.2343 | -189603.6533 | 25.0598** | Proportion | 0.93264 | 0.04910 | 0.01735 | 0.00091 |

*Estimated likelihood values under model A (allowing positive selection) and the null model (no positive selection), likelihood ratio tests (LRTs), and estimated parameters of model A. LRT critical values 3.84 at p = 0.05 (*) and 5.99 at p = 0.01 (**). Branches are as identified in Fig. 2.
The two lineages, seahorses and flatfishes, on which positive selection was most pronounced, have also experienced extraordinary morphological evolution [61, 62]. Thus, it is possible that the positive selection signal detected on mitochondrial OXPHOS genes in these lineages is unrelated to energy demands, but is simply a consequence of rapid genomic evolution in these groups. The probability of nucleotide change is indeed higher in these two lineages than for the other major groups as indicated by five nuclear genes that are not directly involved in OXPHOS system (rag1, rhodopsin, tmo4c4, mhy6, and zic1). However, a positive selection signal was found for only two genes, rag1 and rhodopsin, on one branch leading to flatfishes (LRTs = 72.53, ω = 50.88, three sites 199, 425, and 605 under selection with bayes empirical bayes (BEB) probability of 0.684, 0.501, and 0.958, respectively). Because there was no consistent pattern of selection on these non-OXPHOS genes, it is suggested that the positive selection detected on mitochondrial genes does not reflect a genome-wide pattern of divergence but may be related to adaptation of OXPHOS efficiency.

**Structural position of positively selected sites**

The position of particular amino acids in the tertiary and quaternary structure of a protein may allow inferences about the function of individual residues. In particular, those sites near the catalytic core or other functionally important regions, or those in physical proximity (likely to interact with) other amino acids, would seem most likely to influence protein function.

Complex I performs the first step and is the largest and most complicated enzyme complex in the OXPHOS pathway. It catalyzes the transfer of two electrons from NADH to ubiquinone (Q), coupled to the translocation of four protons across the inner mitochondrial membrane. It is also a major source of reactive oxygen species in mitochondria. Complex I exhibits an L-shaped architecture with a membrane arm and a hydrophilic peripheral arm that protrudes into the mitochondrion (Fig. 3a). The membrane arm consists of 7 core mitochondrial subunits.

The pocket bound by stigmatellin, one OXPHOS inhibitor, is formed by the end of helix C, the helix cd1, the helix ef linker, and the end of helix F. Specific residues of known importance include 271, 275, 125, and 129, 138–153 (indicated in green in Fig. 4). Residues 221 and 194 are also close enough to contact the active site inhibitor [68]. The positively selected sites 194 and 235 (indicated in cyan in Fig. 4) are within these regions, suggesting important functional effects.

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close enough for binding interaction, thereby forming an unusual cross-link structure [69]. One positively selected site 178 is very close to 244 and 240 (Fig. 5b). Two proton translocation pathways, D-pathway and K-pathway, are identified in subunit I. Three key residues of D-pathway 91, 98, and 242, and one key residue of K-pathway 319 are well defined from mutagenesis studies [69] (key residues are indicated in white in Fig. 5b). The positively selected site 178 (indicated in cyan in Fig. 5b) is close to (within 30 Å) the four key residues and could potentially affect proton translocation.

Subunit II (encoded by \( MT-CO2 \) gene) has two transmembrane helices and a hydrophilic beta strand extending to the extra-membrane domain, housing the Cu\(_{A}\) center. The first residue crucial for electron to enter the oxidase complex cytochrome c is 106 (indicated in magenta in Fig. 5c). The other Cu\(_{A}\) liganded residues identified through mutagenesis include 196, 200, 198, 161, 204, and 207 (indicated in magenta in Fig. 5c). One positively selected site 187 is very close to the Cu\(_{A}\) center and the other positively selected site 54 is in a linking strand between two helices of the entry site which includes critical residue 62 (indicated in cyan in Fig. 5c).

Subunit III (encoded by \( MT-CO3 \) gene) is fully embedded in the membrane domain. No key residue of this subunit has been identified through mutagenesis. However, it has been shown that this subunit stabilizes the integrity of the binuclear center in subunit I; for example, when this gene is deleted, only a partially assembled complex results [69].

The sites described above as having potential functional significance were more or less uniformly distributed among branches of the tree and showed no association with particular swimming performance groups. Many sites exhibited evidence of being under positive selection yet did not appear in structural locations that would suggest special functional importance. However, the interaction between amino acids among different proteins or among sites in the same protein is known to have significant functional effects (e.g., [70, 71]). Such interactions could affect protein assembly, stability or specific function. We used 4 Å as the nominal upper limit for weak interactions between amino acid sites as described in [72]. None of the sites identified here as under positive selection were found to be within 4 Å of any other amino acid sites.
Discussion

We examined evidence for adaptation of mitochondrial OXPHOS genes in fishes with different swimming performance. Selection was investigated for specific amino acids sites across the whole phylogenetic tree for these species, as well as for amino acid sites on individual branches of the tree. The results show a strong signal of positive selection on branches in the ancestral parts of the tree, and branches leading to low- and moderate-performance swimmers. However, no evidence of positive selection was observed within clades of the high-performance tunas or billfishes. We did not observe a disproportionate effect of selection on any particular gene as all genes exhibited some positively selected sites but this varied across branches of the tree. We also show that many of the sites identified as being under positive selection occur in structural regions where they may have effects on OXPHOS function. Positively selected sites in other regions may also have functional significance, but their potential effects are less clear.

Our results show the strongest signal of positive selection on branches leading to the lowest performance fishes, while purifying selection was identified on the branches of high performance fishes. This result is in strong contrast to previous studies that have exclusively examined the hypothesized association of positive selection on OXPHOS with increased energetic demands required by higher organismal performance (reviewed in [16]). Our results suggest that a fairly efficient OXPHOS system had evolved under positive selection in the ancestors of the two major groups. Selective modification of OXPHOS on branches leading directly to tunas and billfishes may have further facilitated the evolution of high performance swimming. But once very high performance swimming had evolved in these lineages, purifying selection appears to have predominated on the OXPHOS system as it existed at that time. Conversely, substantial modification due to positive selection occurred in the lower performance lineages of flatfishes and the seahorse + pipefish group. The moderate-high performance jacks and mackerels exhibited moderate-high conservation, with limited positive selection. Thus, the strength of the positive selection signal is inversely proportional to swimming performance within both of these taxonomic groups.

A common expectation is that positive selection should lead to enhanced organismal performance. Moreover, it might be expected that positive selection will lead to increased functional efficiency of OXPHOS in response to the increased ATP demands associated with enhanced performance. In the absence of information on the exact functional significance of individual substitutions, the effects of positive selection to increase or decrease OXPHOS efficiency in these taxa remains unknown.

Fig. 4 Crystal structure of the entire respiratory complex III cytochrome b at 3.16 Å (PDB: 1BCC) from chicken (Gallus gallus). White indicates antimycin-binding cavity formed by helices A (residues 33–54), D (172–204), E (221–245), and the amphipathic surface of helix a (65–72). Green indicates stigmatellin- and myxothiazol-binding pocket formed by residues 271, 275, 125–129, and 138–153. Cyan residues are those identified as positively selected amino acid sites in this study.

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However, our results do not match simple expectations. Positive selection appears to be heterogeneous, fluctuating over phylogenetic time scales, with no simple relationship between the strength of selection and organismal performance. However, one clear pattern is that there is strong functional constraint (negative selection) in these high performance systems and directional selection on these lower performance groups.

Our results appear to contrast with previous studies where positive selection or evolutionary rate variation was found on lineages leading to organisms with high ATP demands. For example, \(MT-CO1\) [73] and \(MT-CO2\) [74] exhibited accelerated \(d_N\) in the lineage leading to hominids that appears to be associated with increased brain size. Grossman et al. [75] found accelerated rate variation for \(MT-CO4\), a nuclear gene in OXPHOS complex IV in catarrhine ancestors of hominids in the period between 18 and 40 Mya and then decelerated in the descendant hominid lineages. On the lineage leading to bats, the only mammals capable of powered flight, eight OXPHOS genes were found to have undergone positive selection [18]. Foote et al. [76] found two positively selected amino acid sites, which could influence overall metabolic performance, in the mitochondrial genes of killer whales (\textit{Orcinus orca}). In addition, the \(MT-ND2\) and \(MT-ND5\) genes of highly migratory Pacific salmon exhibit evidence of positive selection [41].

Our results are consistent with previous studies of the high-performance taxa examined here. Dalziel et al. [39] examined one mitochondrial OXPHOS gene (\(MT-CO2\)) on several branches among high-performance fishes, including billfishes and tunas. They found \(\omega\) was not

\[\text{Fig. 5} \text{ Structure of mitochondrial DNA encoded } MT-CO1 \text{ (red) (also see in 5b), MT-CO2 (yellow) (also see in 5c), and MT-CO3 (green) subunits from bovine heart cytochrome c oxidase at 2.8 Å (PDB: 1OCC) (5a). Amino acid sites in cyan are positively selected sites detected in this study (5a). White indicates key residues in proton translocation pathways: D-pathway and K-pathway in subunit I. Magenta indicates key residue for electron to enter and CuA liganded residues in subunit II.}\]
increased in lineages leading to the tunas but was significantly increased in the lineage preceding the billfish (including several amino acid sites). However, the phylogeny used in [39] does not include the flatfish or sea-horse clades, which are now recognized as close relatives of billfishes and tunas, respectively. Little et al. [40] examined three mitochondrial OXPHOS genes (MT-CO1, 2, and 3) on the single lineage leading to billfishes and found positive selection along that lineage when flatfishes were excluded but no selection was detected when flatfishes were included. The latter result is consistent with our findings and as suggested by Little et al. [40] emphasizes the importance of dense phylogenetic sampling for the analysis of positive selection. We extended these two studies by examining 10 additional mitochondrial OXPHOS genes in the high-performance taxa, and also by explicitly investigating selection on related low performance taxa.

With respect to negative selection on high performance fishes, we speculate that once a reasonably efficient OXPHOS system evolved it may have become difficult to change in the high performance groups. The high performance system might be expected to have a much lower tolerance for non-synonymous substitutions as most (even slight) changes would be likely to have negative functional effects. Conversely, lower performance swimmers may have a much broader tolerance for non-synonymous substitutions because the OXPHOS system is under much lower performance demands. We envision the high performance swimmers as occupying a local optimum on a fitness landscape [77], but their OXPHOS system is so fine-tuned that substitutions that would allow them to cross fitness valleys and reach higher peaks could be strongly deleterious in the short term. On the other hand, lower performance fishes might readily cross such valleys without significant fitness costs because OXPHOS efficiency will be less critical and they may then climb other fitness peaks due to positive selection.

It is also possible that organismal fitness in taxa with low energy demands may be increased by a modified OXPHOS regulatory system or even a reduction in OXPHOS efficiency. In such cases, we would expect to see evidence of positive selection on OXPHOS genes in these taxa. Because there may be trade-offs between OXPHOS rate or efficiency and deleterious effects, reducing OXPHOS efficiency may be adaptive in systems where ATP demand is chronically low. For example, maintenance of a strong chemiosmotic gradient in organisms with low ATP demand may cause increased production of reactive oxygen species (ROS) [38] leading to oxidative stress [78]. Therefore reduction of the H+/2e ratio (increased slippage) due to altered protein structures could be adaptive in low performance species. OXPHOS regulation is highly complex and involves mechanisms independent of structural OXPHOS proteins. Elevated expression of OXPHOS genes and others involved in aerobic respiration could clearly increase aerobic capacity in the absence of selection on specific OXPHOS variants. However, it is clear that positive selection acts on OXPHOS proteins and the effects of positive selection are frequently associated with the evolution of differences in ATP demand.

There are other issues that could affect the efficiency of ATP generation of OXPHOS in high-performance tunas and billfishes. Tunas and billfishes exhibit high metabolic rates to generate ATP to support their high swimming performance (thus high OXPHOS efficiency). However, they are among the few endothermic species of fishes [43, 79, 80] that generate great amount of heat with high metabolic rates (thus reducing ATP generation). Thus, depending on the extent to which body heat is derived from OXPHOS uncoupling, there might be trade-offs between ATP generation and heat generation in these endothermic high-performance fishes. In addition, it has been shown that the heat through mitochondrial proton leak differs among organisms and even differs in cells and tissues in the same organism [38]. It would be helpful to have empirical data about the P/O ratio (how many ATP molecules made from ADP for each oxygen atom consumed), the H+/O ratio (how many protons do mitochondria pump from matrix into inter-membrane space for each oxygen atom consumed), and the H+/ATP ratio (how many protons flow back to the matrix for each ADP molecule phosphorylated to ATP) in these fish species.

High mitochondrial respiration capacity can be achieved through means other than modification of OXPHOS efficiency via genetic variation, such as increased mitochondrial density per tissue mass, more closely packed inner mitochondrial membrane cristae, increased metabolic enzyme activity, increased expression of genes involved in a number of different but related biological pathways such as glycolysis, the Krebs cycle, and fatty acid metabolism. In addition, swimming performance, as in our qualitatively defined groups, may have arisen due to many different morphological and physiological characteristics beyond OXPHOS function. We do not assert that all variation in swimming performance is due to OXPHOS variation, only that some unknown fraction of the performance variation is positively associated with natural selection. A number of recent studies have presented evidence for direct effects of OXPHOS variation on organismal fitness, many with an identified mechanistic basis [81–86]. While these studies employ tractable model organisms such as Drosophila, they provide strong evidence that OXPHOS variation affects organismal performance and fitness in the wild.

In conclusion, we found that patterns of natural selection on mitochondrial OXPHOS genes are complex among fishes with different swimming performance. The type and direction of selection are heterogeneous through...
evolutionary time and vary in ways that would not be readily predicted based solely on organismal performance. The most striking result was extensive positive selection on low-performance swimmers. Although examination of the most recent lineages indicates that positive selection is inversely proportional to organismal performance, the broader pattern indicates a complex interplay between organismal adaptations, ATP demand and OXPHOS function through evolutionary time.

Availability of supporting data
The data sets supporting the results of this article are included in its additional files.

Additional files

Additional file 1: Table S1. Species list with mitochondrial genome accession number from NCBI. Table S2. PCR primers used for nuclear markers used in this study. Table S3. GenBank accession number of nuclear gene (rag1, rag2, tm00404, zic1, myh6, and btb7) sequences of studied species. Figure S1. Phylogeny of six fish groups based on Bayesian and maximum likelihood methods. Figure S2. Summary of selection on all mitochondrial genes. Table S4. Number of amino acid sites showing positive selection signals in each mitochondrial gene on relevant branches. Table S5. Positively selected amino acid sites for each gene on various branches. (DOCX 351 kb)

Abbreviations
MT-CO1: Mitochondrially encoded cytochrome c oxidase I; MT-CO2: Mitochondrially encoded cytochrome c oxidase II; MT-ND1: Mitochondrially encoded NADH dehydrogenase 1; MT-ND2: Mitochondrially encoded NADH dehydrogenase 2; MT-ND3: Mitochondrially encoded NADH dehydrogenase 3; MT-ND4: Mitochondrially encoded NADH dehydrogenase 4; MT-ND4L: Mitochondrially encoded NADH dehydrogenase 4L; MT-ND6: Mitochondrially encoded NADH dehydrogenase 5; MT-NDH: Mitochondrially encoded NADH dehydrogenase 6; MT-CYB: Mitochondrially encoded cytochrome b; MT-ATP6: Mitochondrially encoded ATP synthase 6; MT-ATP8: Mitochondrially encoded ATP synthase 8.

Competing interests
Both authors declare that they have no competing interests.

Authors’ contributions
FZ and RB designed the study; FZ collected and analyzed the data, FZ and RB wrote the manuscript. Both authors read and approved the final manuscript.

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References
1. Rand DM. Thermal habit, metabolic rate and the evolution of mitochondrial DNA. Trends Ecol Evol. 1994;9(4):125–31.
2. Ballard JW, Whittlock MC. The incomplete natural history of mitochondria. Mol Ecol. 2004;13(4):729–44.
3. da Fonseca RR, Johnson WE, O’Brien SJ, Ramos MJ, Antunes A. The adaptive evolution of the mammalian mitochondrial genome. BMC Genet. 2008;9(1):119.
4. Ballard J, Melvin R. Linking the mitochondrial genotype to the organismal phenotype. Mol Ecol. 2010;19(8):1523–39.
5. Hill GE. Cellular respiration: the nexus of stress, condition, and ornamentation. Integr Comp Biol. 2014;54(6):645–57.
6. Ballard JW, Rand DM. The population biology of mitochondrial DNA and its phylogenetic implications. Annu Rev Ecol Syst. 2005;621–642.
7. Wallace DC. Mitochondrial DNA mutations in disease and aging. Environ Mol Mutagen. 2010;51(5):440–50.
8. Mishmar D, Ruiz-Pesini E, Golik P, Macaulay V, Clark AG, Hosseini S, et al. Natural selection shaped regional mtDNA variation in humans. Proc Natl Acad Sci. 2003;100(1):171–6.
9. Rand D, Haney R, Fry A. Cytochrome c oxidase: the genomics of cooperation. Trends Ecol Evol. 2004;19(12):645–53.
10. Ruiz-Pesini E, Mishmar D, Brandon M, Procaccio V, Wallace DC. Effects of purifying and adaptive selection on regional variation in human mtDNA. Science. 2004;303(5655):223–6.
11. Moyer GR, Winemiller KO, McPhee MV, Turner TF. Historical demography, selection, and coalescence of mitochondrial and nuclear genes in Prochilodus species of northern South America. Evolution. 2005;59(3):599–610.
12. Bazin E, Géminis S, Galtier N. Population size does not influence mitochondrial genetic diversity in animals. Science. 2006;31(5773):570–2.
13. Ballard JW, Melvin RG, Kataeva SD, Maas K. Mitochondrial DNA variation is associated with measurable differences in life-history traits and mitochondrial metabolism in Drosophila simulans. Evolution. 2007;61(7):1735–47.
14. Meklejohn CD, Montooth KL, Rand DM. Positive and negative selection on the mitochondrial genome. Trends Genet. 2007;23(6):259–63.
15. Hassanin A, Ropiquet A, Couloux A, Crusad C. Evolution of the mitochondrial genome in mammals living at high altitude: new insights from a study of the tribe Caprini (Bovidae, Antilopinae). J Mol Evol. 2009;68(4):293–310.
16. Garvin MR, Belawski JP, Sazanov LA, Gharrett AJ. Review and meta-analysis of natural selection in mitochondrial complex I in metazoa. J Zool Syst Evol Res. 2015;53(1):1–17.
17. Doan JW, Schmidt TR, Wildman DE, Uddin M, Goldberg A, Hütttemann M, et al. Coadaptive evolution in cytochrome c oxidase: 9 of 13 subunits show accelerated rates of nonsynonymous substitution in anoploist pantropical. Mol Phylogenet Evol. 2004;33(3):944.
18. Shen Y-Y, Liang L, Zhu Z-H, Zhou W-P, Irwin DM, Zhang Y-P. Adaptive evolution of energy metabolism genes and the origin of flight in bats. Proc Natl Acad Sci. 2010;107(19):8666–71.
19. Welch AI, Bedoya-Reina OC, Carretero-Paulet L, Miller W, Rode KD, Lindqvist C. Polar bears exhibit genome-wide signatures of bioenergetic adaptation to life in the Arctic environment. Genome Biol Evol. 2014;6(2):433–50.
20. Beamish F. Swimming capacity. New York: Academic; 1978.
21. Block BA, Booth D, Carey FG. Direct measurement of swimming speeds and depth of blue marlin. J Exp Biol. 1992;166(1):267–84.
22. Lauver GV. Fish locomotion: recent advances and new directions. Ann Rev Mar Sci. 2015;7:521–45.
23. Blake R. Energetics of leaping in dolphins and other aquatic animals. J Mar Biol Assoc U K. 1983;61(9):2087.
24. Vogel S. Life in moving fluids: the physical biology of flow. Princeton: Princeton University Press; 1994.
25. Webb PW. The biology of fish swimming, mechanics and physiology of animal swimming. 1994. p. 4562.
26. Videler J, Nolet B. Costs of swimming measured at optimum speed: scale effects, differences between swimming styles, taxonomic groups and submerged and surface swimming. Comp Biochem Physiol A Physiol. 1990;97(2):91–9.
27. Wainwright PC, Bellwood DR, Westneat MW. Ecomorphology of locomotion in labrid fishes. Environ Biol Fish. 2002;65(1):47–62.
28. Walker JA, Westneat MW. Performance limits of labriform propulsion and correlates with fin shape and motion. J Exp Biol. 2002;205(2):177–87.
29. Graham J, Koehn F, Dickson K. Distribution and relative proportions of red muscle in scorbutid fishes: consequences of body size and relationships to locomotion and endothermy. Can J Zool. 1983;61(9):2087–96.
53. Singh M, Gupta A, Laka W. In silico 3-D structure prediction of cytochrome b protein of sisorid catfish Glytostrodon ngapang. Indian J Biotechnol. 2012;11(2):156–62.
54. Yoshikawa S, Shinzawa-itoh K, Nakashima R, Yano R, Yamashita E, Inoue N, et al. Redox-coupled crystal structural changes in bovine heart cytochrome c oxidase. Science. 1998;280(5370):7123–9.
55. Tsukihara T, Aoyama H, Yamasaki E, Tomizaki T, Yamaguchi H, Shinzawa-itoh K, et al. The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 Å. SCIENCE. 1996;272:1136–44.
56. Benjamini Y. Discovering the false discovery rate. J R Stat Soc Ser B (Stat Methodol). 2010;72(4):405–16.
57. Wilson A, Ori J. The evolutionary origins of Syngnathidae: pipefishes and seahorses. J Fish Biol. 2011;78(6):1603–23.
58. Yin M, Blaxter J. Morphological changes during growth and starvation of larval cod (Gadus morhua L.) and flounder (Platichthys flesus L.). J Exp Mar Biol Ecol. 1986;104(1):215–28.
59. Campos Y, Martin RA, Rubio JC, Olmo MC, Cabello A, Arenas J. Bilateral strait necrosis and NELAS associated with a new T3308C mutation in the mitochondrial ND1 gene. Biochem Biophys Res Commun. 1997;238(3):323–5.
60. Osdal SH, Engeland T, Musse MA, Rognum TO. Possible role of mtDNA mutations in sudden infant death. Pediatr Neurosci. 2002;27(1):123–9.
61. Simon DK, Friedman J, Breakefield XO, Jankovic J, Brin MF, Provias J, et al. A heteroplasmonic mitochondrial complex I gene mutation in adult-onset dystonia. Neurogenetics. 2003;4(4):199–205.
62. Etterov R, Sazanov LA. Structure of the membrane domain of respiratory complex I. Nature. 2011;476(7361):1414–20.
63. Scheffler I. Mitochondrial electron transport and oxidative phosphorylation. Mitochondria. 1999;141:245.
64. Zhang Z, Huang L, Shulmeister VM, Chi Y-L, Kim KH, Hung L-W, et al. Electron transfer by domain movement in cytochrome bc1. Nature. 1998;392(6677):677–84.
65. Richter DM, Ludwig B. Cytochrome c oxidase—structure, function, and physiology of a redox-driven molecular machine, Reviews of physiology, biochemistry and pharmacology. Berlin: Springer; 2003. p. 47–74.
66. Gensini M, Fuchs A, Shani N, Fridman Y, Corral-Debrinski M, Abaroni A, et al. Coevolution predicts direct interactions between mtDNA-encoded and nDNA-encoded subunits of oxidative phosphorylation complex I. J Mol Biol. 2010;404(1):158–71.
67. Willett CS, Burton RS. Environmental influences on epistatic interactions: viabilities of cytochrome c genotypes in interpopulation crosses. Evolution. 2003;57(10):2286–92.
68. Schmidt TR, Wu W, Goodman M, Grossman LI. Evolution of nuclear and mitochondrial-encoded subunit interaction in cytochrome c oxidase. Mol Biol Evol. 2001;18(4):563–9.
69. Andrews TD, Eastal S. Evolutionary rate acceleration of cytochrome c oxidase subunit I in similar primates. J Mol Evol. 2000;50(6):562–8.
70. Adkins RM, Honeycutt RL. Evolution of the primate cytochrome c oxidase subunit II gene. J Mol Evol. 1994;38(3):215–31.
71. Grossman LI, Schmidt TR, Wildman DE, Goodman M. Molecular evolution of aerobic energy metabolism in primates. Mol Phylogenet Evol. 2001;18(1):26–36.
72. Foote AD, Morin PA, Durban JW, Pitman RL, Kidd J, Willerslev E, et al. Positive selection on the killer whale mtgenome. Biol Lett. 2011;7(1):116–8.
73. Gavrilets S. Evolution and speciation on holey adaptive landscapes. Trends Ecol Evol. 2003;18(10):R453–66.
74. Meiklejohn CD, Holmbeck MA, Siddiq MA, Abt DN, Rand DM, Montooth KL. The evolutionary origins of the scombridae (tunas and mackerels): members of a trans-subordinal origins of endothermy in teleost fishes. Mol Phylogenet Evol. 2010;404(1):158–71.
75. Grossman LI, Schmidt TR, Wildman DE, Goodman M. Molecular evolution of aerobic energy metabolism in primates. Mol Phylogenet Evol. 2001;18(1):26–36.
76. Foote AD, Morin PA, Durban JW, Pitman RL, Kidd J, Willerslev E, et al. Positive selection on the killer whale mtgenome. Biol Lett. 2011;7(1):116–8.
77. Gavrilets S. Evolution and speciation on holey adaptive landscapes. Trends Ecol Evol. 2003;18(10):R453–66.
78. Meiklejohn CD, Holmbeck MA, Siddiq MA, Abt DN, Rand DM, Montooth KL. The evolutionary origins of the scombridae (tunas and mackerels): members of a trans-subordinal origins of endothermy in teleost fishes. Mol Phylogenet Evol. 2010;404(1):158–71.
79. Block BA, Finnerty JR, Stewart AF, Kidd J. Evolution of endothermy in fish: mapping physiological traits on a molecular phylogeny. Science. 1993;260:210–4.
80. Dickson KA, Graham JB. Evolution and consequences of endothermy in teleosts, the tunas. Can J Zool. 1991;69(7):2002.
81. Montooth KL, Abt DN, Hofmann J, Rand DM, Comparative genomics of Drosophila mtDNA novel features of conservation and change across functional domains and lineages. J Mol Evol. 2009;69(3):134–141.
82. Pichaud N, Ballard JWO, Tanguay RM, Blier PU. Mitochondrial haplotype diversity affects specific temperature sensitivity of mitochondrial respiration. J Eukaryot Microbiol. 2013;65(3):13–26.
83. Mlekjo John CD, Holmbeck MA, Siddiq MA, Abt DN, Rand DM, Montooth KL. An incompatibility between a mitochondrial RNA and its nuclear-encoded RNA synthetase compromises development and fitness in Drosophila. PLoS Genet. 2013;9(1):e1003238.
84. Pichaud N, Ballard JWO, Tanguay RM, Blier PU. Naturally occurring mitochondrial DNA haplotypes exhibit metabolic differences: insight into functional properties of mitochondria. Evolution. 2012;66(10):3189–97.

85. Cooper BS, Burnus C, Ji C, Hahn MW, Montooth K. Similar efficacies of selection shape mitochondrial and nuclear genes in Drosophila melanogaster and Homo sapiens. BioRxiv. 2014. doi:10.1101/010355.

86. Greenlee KJ, Montooth KL, Helm BR. Predicting performance and plasticity in the development of respiratory structures and metabolic systems. Integr Comp Biol. 2014;54(2):307–22.