Preliminary assessment of adaptive evolution of mitochondrial protein coding genes in darters (Percidae: Etheostomatinae) [version 2; peer review: 1 not approved]

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Abstract
Background: Mitochondrial DNA of vertebrates contains genes for 13 proteins involved in oxidative phosphorylation. Some of these genes have been shown to undergo adaptive evolution in a variety of species. This study examines all mitochondrial protein coding genes in 11 darter species to determine if any of these genes show evidence of positive selection.

Methods: The mitogenome from four darter was sequenced and annotated. Mitogenome sequences for another seven species were obtained from GenBank. Alignments of each of the protein coding genes were subject to codon-based identification of positive selection by Selecton, MEME and FEL.

Results: Evidence of positive selection was obtained for six of the genes by at least one of the methods. \textit{CYTB} was identified as having evolved under positive selection by all three methods at the same codon location.

Conclusions: Given the evidence for positive selection of mitochondrial protein coding genes in darters, a more extensive analysis of mitochondrial gene evolution in all the extant darter species is warranted.

Keywords
Mitogenome, mtDNA, adaptive evolution, positive selection, Etheostoma, Percina

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Author roles: Kral LG: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing – Original Draft Preparation; Watson S: Data Curation, Formal Analysis, Investigation, Methodology

Competing interests: No competing interests were disclosed.

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Introduction
Mitochondria provide cells with nearly all energy as a result of oxidative phosphorylation (OXPHOS). Vertebrate mitochondria are primarily maternally inherited and contain mitochondrial DNA (mtDNA), which contains 13 protein coding genes, the products of which contribute, along with nuclear-encoded proteins (Sumnucks et al., 2017), to the formation of the mitochondrial OXPHOS machinery (Ingman & Gyllensten, 2006; Ladoukakis & Zouros, 2017). While purifying selection acts on deleterious mtDNA mutations (Burr et al., 2018), James et al. (2016) utilized a variety of McDonald-Kreitman type analyses of a large number of animal species to show that mtDNA evolution is dominated by slightly deleterious mutations but also undergoes a significant amount of adaptive evolution. Several studies of the evolution of mtDNA-encoded mitochondrial protein coding genes both within fish species (Consuegra et al., 2015; Teacher et al., 2012) and between fish species (D’Anatro et al., 2017; Garvin et al., 2011; Zhang & Broughton, 2015) identified positive selection acting on some of these genes.

Darters are a group of approximately 200 small, benthic perch-like fish species that are found in streams and rivers in eastern North America (Near et al., 2011). Various species are adapted to fast water or slow water habitats that can differ in oxygen content, especially in the summer, and these species differ in their metabolic rates (Kist, 2016; Ultsch et al., 1978). Adaptation to such environmental niches may include adaptive changes to the OXPHOS machinery and thus this study utilizing all currently available mitochondrial genome sequences of darter species was carried out to determine if positive selection can be detected in any of the mitochondrial protein coding genes.

Methods
Mitochondrial genomes
All mitochondrial genome sequences utilized in this study were obtained from GenBank except those from Etheostoma chuckwachatte, Etheostoma jessiae, Etheostoma spectabile, Etheostoma tallapoosae and Percina crypta. The E. spectabile sequence was obtained from Rachel Moran, University of Illinois. Whole genome sequencing was performed on E. chuckwachatte, E. jessiae, E. tallapoosae and P. crypta genomic DNA (purified from fin/muscle tissue with the Qiagen DNeasy Blood and Tissue Kit) by the Georgia Genomic Facility at the University of Georgia utilizing the Illumina NextSeq (PE 150) or MiSeq (PE 250) sequencer. A subset of sequence reads from each species, sufficient for at least 30-fold coverage, was aligned to one of the GenBank obtained darter mitochondrial genomes utilizing the Map to Reference function in Geneious 9.1.8 software (Biomatters Ltd., Auckland, New Zealand). Annotated darter mitochondrial genome sequences from GenBank were aligned with the newly assembled darter mitochondrial genome sequences and annotations were transferred and manually adjusted where necessary with Geneious software.

Phylogenetic analysis
Phylogenetic analysis was carried out on all 13 concatenated and aligned mitochondrial protein coding sequences of the 11 darter species as well as of Perca flavesca, Sander vitreus and Anarchichas minor (outgroup). Bayesian inference (BI) analysis was performed with MrBayes (Ronquist & Huelsenbeck, 2003), as implemented in Geneious software but utilizing optimal partitioning and optimal substitution models determined by PartitionFinder v2.1.1 (AICc, greedy algorithm, MrBayes specific models) (Guindon et al., 2010; Lanfear et al., 2012; Lanfear et al., 2017). The rooted darter sub-tree (Figure 1) was extracted with MEGA 7.0.26 software (Kumar et al., 2016). Aligned darter mitochondrial protein coding genes were subject to site-specific tests for positive selection utilizing the Selecton implementation of an empirical Bayes approach (Doron-Faigenboim et al., 2005; Stern et al., 2007) and also the MEME and FEL methods implemented on the Datamonkey webserver (Delport et al., 2010). The darter tree was used in all three of these analyses.

Results and discussion
Identification of codons under positive selection
Codons under positive selection were identified in seven of the thirteen mitochondrial protein coding genes by at least one of the methods utilized. Specifically, as shown in Table 1, the MEME method identified two codons in COX1, two codons in CYTB, three codons in ND2, and one codon each in ND3 and in ND5. The FEL method identified one codon in COX1, CYTB and ND3. The codons identified as being under positive selection by the FEL method were also identified as being under positive selection by MEME method. Specifically, codon 489 in COX1, codon 96 in CYTB and codon 9 in ND3. As shown in Table 2, the Selecton method identified one codon in ATP6, one codon in COX3, one codon in CYTB and two codons in ND5. While codons under positive selection were identified in ND5 by MEME and Selecton, these methods did not identify the same codons. Specifically, codon 32 was identified in ND5 by MEME and codons 479 and 573 were identified by Selecton. Only codon 96 in CYTB was identified as being under positive selection by all three methods.

As shown in Table 3, The changes in codon 96 of CYTB are conservative substitutions of nonpolar amino acids Methionine and Leucine. Similarly, codon 82 of CYTB, codons 119 and 327 of ND2, codon 9 of ND3 and codons 479 and 573 of ND5 also involve substitutions of only nonpolar amino acids. Codon 32 of ND5 involves the conservative substitution of basic amino acids where lysine is present in the Percina species while Arginine is present in the Etheostoma species. While these conservative substitutions were identified as sites under positive selection by the statistical analyses utilized, it is possible that these sites may simply be under relaxed selective stringency. The remaining sites involve substitutions of nonpolar amino acids and polar amino acids threonine and serine (Table 3). These substitutions may have a greater effect upon
Figure 1. Darter phylogenetic tree produced using Bayesian estimation (Mr. Bayes) of concatenated mitochondrial protein coding genes. Branch lengths measured in number of substitutions per site. Posterior probabilities of all branches are greater than 0.91. Pm, Percina macrolepida; Pc, Percina crypta; Ecw, Etheostoma chuckwachatta; Ec, Etheostoma caeruleum; Er, Etheostoma radiosum; Eo, Etheostoma okaloosae; Es, Etheostoma spectabile; Ejs, Etheostoma jessiae; En, Etheostoma nigrum; Ez, Etheostoma zonale; Et, Etheostoma tallapoosae.

Table 1. Genes and sites inferred to be under positive selection by MEME and FEL. Default significance cutoff value of p<0.1 was used.

<table>
<thead>
<tr>
<th>Gene</th>
<th>MEME</th>
<th>FEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td>p value</td>
<td>Site</td>
</tr>
<tr>
<td>COX1</td>
<td>3</td>
<td>0.04</td>
</tr>
<tr>
<td>489</td>
<td>0.06</td>
<td>489</td>
</tr>
<tr>
<td>CYTB</td>
<td>82</td>
<td>0.09</td>
</tr>
<tr>
<td>96</td>
<td>0.02</td>
<td>96</td>
</tr>
<tr>
<td>ND2</td>
<td>119</td>
<td>0.02</td>
</tr>
<tr>
<td>327</td>
<td>0.06</td>
<td>-</td>
</tr>
<tr>
<td>ND3</td>
<td>32</td>
<td>0.06</td>
</tr>
<tr>
<td>ND5</td>
<td>32</td>
<td>0.08</td>
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</table>
the structure/function of the relevant OXPHOS complexes. However, the only way to be certain that any of these substitutions identified as being under positive selection are physiologically meaningful, will be to assess the physiochemical functions of these complexes directly.

### Analysis of sites under positive selection

While the Bayesian estimates of the Ka/Ks ratio of the sites identified by Selecton are greater than 1, the lower bounds of the confidence intervals defined by the 5th and 95th percentiles of the posterior distribution inferred for these sites are less than 1. Therefore, the reliability of positive selection of these sites identified by Selecton is not very strong. However, positive selection of the four proteins identified by Selecton has been found to be significant (p = 0.001) by the likelihood ratio test of log-likelihood for model M8 (allowing positive selection) versus model M8a (not allowing positive selection).

### Drivers of selection

These results indicate that it is likely that the evolution of various darter lineages included positive selection of at least some of the mitochondrial encoded OXPHOS machinery variants. Presumably, this positive selection would have been driven by adaptation to specific environmental factors. As an example of mitochondrial adaptation to environmental factors, Ma et al. (2015) found that adaptation of Chinese glyptosternoid fishes to high-elevation of the Tibetan Plateau is correlated to signals of positive selection of the Cox1 gene. That adaptation to hypoxia is correlated to physiological adaptation of the OXPHOS machinery has been demonstrated by O₂ binding kinetics of cytochrome c oxidase (COX) in hypoxia tolerant vs. intolerant sculpin species (Lau et al., 2017). In this sculpin study, several nonsynonymous substitutions in COX3 were identified by in silico analysis as candidates for explaining the adaptive variation of COX O₂ binding. That mitochondrial haplotype variants are

<table>
<thead>
<tr>
<th>Species</th>
<th>ATP6 Site</th>
<th>COX1 Sites</th>
<th>COX3 Site</th>
<th>CYTB Sites</th>
<th>ND2 Sites</th>
<th>ND3 Site</th>
<th>ND5 Sites</th>
</tr>
</thead>
<tbody>
<tr>
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<td>ACA-Thr</td>
<td>ACC-Thr</td>
<td>TCA-Ser</td>
<td>GGA-Gly</td>
<td>CTT-Leu</td>
<td>CTG-Leu</td>
<td>AAA-Lys</td>
</tr>
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<td>ATT-Ile</td>
<td>ACC-Thr</td>
<td>ACA-Gly</td>
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<td>TTT-Leu</td>
<td>CTT-Leu</td>
<td>ATT-Ile</td>
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<tr>
<td>Eow</td>
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<td>ACC-Thr</td>
<td>ACC-Thr</td>
<td>AGT-Met</td>
<td>CGT-Leu</td>
<td>TTA-Leu</td>
<td>GTA-Val</td>
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<tr>
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<td>ACC-Thr</td>
<td>ACC-Gal</td>
<td>GCT-Ala</td>
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<tr>
<td>Er</td>
<td>GCT-Ala</td>
<td>ACT-Thr</td>
<td>ACT-Thr</td>
<td>ATT-Ile</td>
<td>TTC-Leu</td>
<td>ACC-Thr</td>
<td>AGC-Ala</td>
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<tr>
<td>Eos</td>
<td>ATA-Met</td>
<td>ACT-Met</td>
<td>ACT-Thr</td>
<td>ACT-Leu</td>
<td>AGC-Ala</td>
<td>GTC-Val</td>
<td>TCA-Leu</td>
</tr>
<tr>
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<td>GTT-Gly</td>
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<td>ATG-Met</td>
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<td>CTT-Leu</td>
<td>GCC-Thr</td>
<td>GTA-Val</td>
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<tr>
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<td>AGT-Leu</td>
<td>CTT-Leu</td>
<td>TTA-Leu</td>
<td>TTA-Leu</td>
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</table>

### Table 2. Genes and sites inferred to be under positive selection by Selecton

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position</th>
<th>Ka/Ks</th>
<th>CI</th>
<th>M8 likelihood</th>
<th>M8a likelihood</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP6</td>
<td>64</td>
<td>1.4</td>
<td>(0.26, 4.9)</td>
<td>-3537.33</td>
<td>-3545.94</td>
<td>0.001</td>
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<tr>
<td>COX3</td>
<td>44</td>
<td>4.8</td>
<td>(0.26, 4.9)</td>
<td>-3320.11</td>
<td>-3342.84</td>
<td>0.001</td>
</tr>
<tr>
<td>CYTB</td>
<td>96</td>
<td>1.1</td>
<td>(0.26, 4.9)</td>
<td>-5469.81</td>
<td>-5498.22</td>
<td>0.001</td>
</tr>
<tr>
<td>ND5</td>
<td>479</td>
<td>1.9</td>
<td>(0.31, 4.9)</td>
<td>-9664.27</td>
<td>-9683.6</td>
<td>0.001</td>
</tr>
</tbody>
</table>

### Table 3. Nucleotide substitutions and amino acid substitutions at codon sites inferred to be under positive selection by MEME, FEL and/or Selecton

<table>
<thead>
<tr>
<th>Species</th>
<th>ATP6 Site</th>
<th>COX1 Sites</th>
<th>COX3 Site</th>
<th>CYTB Sites</th>
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<td>ACT-Thr</td>
<td>ATT-Ile</td>
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<td>Eos</td>
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<td>ACT-Met</td>
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<td>ACC-Thr</td>
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<tr>
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<td>TTA-Leu</td>
</tr>
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</table>
subject to selection by environmental factors has been demonstrated experimentally in *Drosophila*. When a mixed population of “cold adapted” haplogroups and “warm adapted” haplogroups were maintained for a number of generations at differing temperatures, the frequency of the “cold adapted” haplogroup increased at the cooler temperature and decreased at the warmer temperature (Lajbner et al., 2018). Given the results indicating positive mitochondrial gene selection in this sampling of darter species, it would seem that a more expansive analysis of the evolution of mitochondrial protein coding genes in the approximately 200 extant darter species (Near et al., 2011) may be warranted to identify lineage-specific adaptations and, potentially, their correlations to relevant life history traits once those putative adaptations are verified to have altered the physiochemical properties of the OXPHOS machinery.

Data availability

Underlying data

All mitogenome sequences are available in GenBank.

**Percina macrolepida**: accession number DQ536430, [https://identifiers.org/ncbiprotein:DQ536430](https://identifiers.org/ncbiprotein:DQ536430).

**Percina crypta**: accession number KY965073, [https://identifiers.org/ncbiprotein:KY965073](https://identifiers.org/ncbiprotein:KY965073).

**Etheostoma chuckwachatte**: accession number KY965071, [https://identifiers.org/ncbiprotein:KY965071](https://identifiers.org/ncbiprotein:KY965071).


Acknowledgements

Special thanks to Rachel Moran for access to the *E. spectabile* mitogenome sequence, to Frank Fontanella for guidance in the use of Mr. Bayes software, and the Warm Springs Fish Technology Center for darter specimens.

References


Open Peer Review

Current Peer Review Status: X

Version 1

Reviewer Report 09 May 2019

https://doi.org/10.5256/f1000research.19195.r47213

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Dusan Kordis
Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Ljubljana, 1000, Slovenia

The authors tried to find the evidence of positive selection in 13 mitochondrial proteins, involved in oxidative phosphorylation, in 11 species of darters. Darters are the second largest family of North American fishes and only the minnows (Cyprinidae) have more species. Kral and Watson have found some evidence for positive selection acting on 6 mitochondrial genes, by at least one method used (M8 in Selecton, FEL and MEME in Datamonkey). However, they found that only CYTB gene has evolved under positive selection by all three methods used. The authors expect that a more extensive analysis of mitochondrial gene evolution in all extant darter species may identify lineage specific adaptations and their contributions to the relevant life history traits.

I found a number of problems in the present version of the manuscript:

1. Biology and some special adaptations of darters should be explained in the Introduction. Are there any differences between darter species regarding their life styles, ecology, physiology, metabolism etc.? This information can be used in the interpretation of the observed positive selection in some mitochondrial genes.

2. The authors have just listed the codon positions that have been found to be under positive selection. From the codon numbers, it is very difficult to see what the impact of positively selected sites is. It could be actually biologically important or meaningless.

3. Therefore, the authors must use 3D models of the analysed mitochondrial proteins to show the consequences of the positively selected sites (PSS)/amino acids on the protein secondary and tertiary structure. These amino acids may be functionally important or neutral. The authors should follow some good examples of such analyses (e.g. da Fonseca et al., 2008, Castoe et al., 2009).

4. The authors should indicate the chemical nature of amino acids that are encoded by positively selected codons. Some particular polar amino acids are highly mutable and may not be associated with positive selection (e.g. Xia and Kumar, 2006).
5. The authors should compare their results (e.g. PSS) with the data from literature (e.g. from fishes and other vertebrates) to demonstrate that some positions are conserved PSS or are unique/different PSS.

6. Discussion is very poor and should be strongly improved by obtaining new data about the locations of PSS on the 3D models of the analysed mitochondrial genes. In such a way, their data can be put into biological perspective.

7. The title is uncompelling, merely indicating that the authors haven't obtained any serious conclusions.

References

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: evolutionary genomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.
Leos Kral, University of West Georgia, Carrollton, USA

Thank you for your review and suggestions. I have made revisions to address some of them and I am also providing rationale here for not addressing others.

To address point 1, the introduction was expanded to include the most likely difference between darter species that may lead to OXPHOS genes adaptation.

To address points 2 and 4, table 3 has been added and the results discussion expanded accordingly.

Points 3, 5 and 6 were not incorporated into revision for several reasons. Firstly, I do not have the necessary expertise to confidently relate amino acid changes in protein 3D structure to biochemical function and any such discussion would be only speculative. Furthermore, such an analysis would not necessarily determine if a particular substitution is functionally important or neutral. Work carried out on directed evolution of proteins has demonstrated that mutations that influence the evolution of a protein are not obviously identified as such from a structural analysis since such mutations do not always alter the catalytic sites (see references below). I believe that the Ka/Ks analysis is only indicative of possible adaptive changes and should be first verified by direct assessment of the function of the proteins. I have incorporated this view into the revision.

Point 7. The title is a truthful representation of the study. The number of species for which mtDNA genome sequences are available is just large enough to meet the minimum number of species acceptable for Ka/Ks analysis (at least for Selecton). The scope of this study was simply to ascertain if adaptive evolution can be detected to warrant further study. This would include verification of actual changes in OXPHOS function suggested by Ka/Ks analysis.

References:


Competing Interests: No competing interests were disclosed.
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