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RESEARCH ARTICLE

Preliminary assessment of adaptive evolution of mitochondrial protein coding genes in darters (Percidae: Etheostomatinae) [version 1; 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. *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

Open Peer Review

Approval Status

	1	2
version 2 (revision) 18 Oct 2019	 view	 view
version 1 14 Apr 2019	 view	

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Ljubljana, Slovenia

2. **Dongsheng Zhang**, Shanghai Ocean
University, Shanghai, China

Any reports and responses or comments on the article can be found at the end of the article.

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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

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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 (Sunnucks *et al.*, 2017), to the formation of the mitochondrial OXPHOS machinery (Ingman & Gyllenstein, 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. This study utilized all currently available mitochondrial genome sequences of darter species to determine if positive selection can be detected in any of the mitochondrial protein coding genes. Such positive selection would imply that adaptation of the OXPHOS machinery to differing environmental niches had occurred in at least some lineages during darter speciation.

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 flavescens*, *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 (Delpont *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.

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 mitochondrially 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 O_2 binding kinetics of cytochrome c oxidase (COX) in hypoxia tolerant vs. intolerant

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 macrolepidia*; Pc, *Percina crypta*; Ecw, *Etheostoma chuckwachatte*; 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.

Gene	MEME		FEL	
	Site	p value	Site	p value
COX1	3	0.04	-	-
	489	0.06	489	0.04
CYTB	82	0.09	-	-
	96	0.02	96	0.05
ND2	119	0.02	-	-
	327	0.06	-	-
	339	0.05	-	-
ND3	9	0.06	9	0.05
ND5	32	0.08	-	-

Table 2. Genes and sites inferred to be under positive selection by Selecton. Ka/Ks ratio indicates the ratio of nonsynonymous/synonymous mutation rates, CI is the confidence interval of the Ka/Ks ratio defined by the 5th and 95th percentiles of the posterior distribution inferred for the position. The p value is the significance of the likelihood ratio test between the M8 model (selection allowed) vs. M8a model (selection not allowed) of the gene.

Gene	Position	Ka/Ks	CI	M8 likelihood	M8a likelihood	p value
ATP6	64	1.4	(0.26, 4.9)	-3537.33	-3545.94	0.001
COX3	44	4.8	(0.26, 4.9)	-3320.11	-3342.84	0.001
CYTb	96	1.1	(0.26, 4.9)	-5469.81	-5498.22	0.001
ND5	479	1.9	(0.31, 4.9)	-9664.27	-9683.6	0.001
	573	1.4	(0.31, 4.9)			

sculpin species (Lau *et al.*, 2017). In this study, several non-synonymous 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 subject 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 preliminary results indicating positive mitochondrial gene selection in darters, 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.

Data availability

Underlying data

All mitogenome sequences are available in GenBank.

Percina macrolepidia: accession number DQ536430, <https://identifiers.org/ncbigi/gi:105873653>.

Percina crypta: accession number KY965073, <https://identifiers.org/ncbigi/gi:1238789905>.

Etheostoma chuckwachatte: accession number KY965071, <https://identifiers.org/ncbigi/gi:1238789877>.

Etheostoma caeruleum: accession number KY660678, <https://identifiers.org/ncbigi/gi:1229242602>.

Etheostoma radiosum: accession number AY341348, <https://identifiers.org/ncbigi/gi:48762649>.

Etheostoma okaloosae: accession number KY747492, <https://identifiers.org/ncbigi/gi:1215348329>.

Etheostoma spectabile: accession number MK243404, <https://identifiers.org/ncbigi/gi:1605131112>.

Etheostoma jessiae: accession number KY965072, <https://identifiers.org/ncbigi/gi:1605131112>.

Etheostoma nigrum: accession number KT289926, <https://identifiers.org/ncbigi/gi:1035438550>.

Etheostoma zonale: accession number AP005994, <https://identifiers.org/ncbigi/gi:1173213864>.

Etheostoma tallapoosae: accession number KY952221, <https://identifiers.org/ncbigi/gi:1236181000>.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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.

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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.*, 2008¹, Castoe *et al.*, 2008²)
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, 2006³).

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 unconvincing, merely indicating that the authors haven't obtained any serious conclusions.

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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.

Author Response 07 Oct 2019

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.

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Competing Interests: No competing interests were disclosed.

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