RESEARCH ARTICLE

REVISED Preliminary assessment of adaptive evolution of mitochondrial protein coding genes in darters (Percidae: Etheostomatinae) [version 2; peer review: 1 approved, 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

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

1. **Dusan Kordis**, Jožef Stefan Institute, 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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REVISED Amendments from Version 1

Introduction was expanded to include information about darters and their adaptation to different niches that may select for OXPHOS variants. Also, [Table 3](#) was added to specify types of substitutions identified as being under positive selection and the results and discussion section was expanded to incorporate this addition.

Any further responses from the reviewers can be found at the end of the article

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.

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 flavescens*, *Sander vitreus* and *Anarhichas 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.

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 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)			

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

Species	ATP6 Site	COX1 Sites		COX3 Site	CYTB Sites		ND2 Sites			ND3 Site	ND5 Sites		
	64	3	489	44	82	96	119	327	339	9	32	479	573
Pm	ACA-Thr	ACC-Thr	TCA-Ser	GGA-Gly	CTT-Leu	ATG-Met	CTT-Leu	ATA-Met	ACC-Thr	ATT-Ile	AAA-Lys	ATT-Ile	ATA-Met
Pc	ATT-Ile	ACC-Thr	TCA-Ser	GGA-Gly	CTT-Leu	TTG-Leu	CTT-Leu	ATA-Met	ACC-Thr	GCT-Ala	AAG-Lys	CTC-Leu	GTA-Val
Ecw	ACA-Thr	ACC-Thr	ACA-Thr	ACC-Thr	ATG-Met	CTG-Leu	CTT-Leu	CTC-Leu	TTA-Leu	GTT-Val	CGA-Arg	ATT-Ile	GTC-Val
Ec	ACC-Thr	ACC-Thr	GCA-Ala	GCT-Ala	ATT-Ile	ATG-Met	CTT-Leu	GTC-Val	ACC-Thr	GTT-Val	CGA-Arg	ATT-Ile	ATT-Ile
Er	GCT-Ala	ACC-Thr	ACA-Thr	ACT-Thr	ATT-Ile	TTG-Leu	CTT-Leu	ATC-Ile	GCC-Ala	GTT-Val	CGA-Arg	ACG-Thr	CTT-Leu
Eo	ATA-Met	ATC-Met	ACA-Thr	ACC-Thr	CTT-Leu	ATG-Met	CTT-Leu	CTC-Leu	ACC-Thr	GTT-Val	CGA-Arg	TCC-Ser	ATC-Ile
Es	GTC-Val	ATC-Met	GCA-Ala	GGC-Gly	CTT-Leu	CTT-Leu	ATG-Met	CTT-Leu	GCC-Ala	CTT-Leu	CGA-Arg	CTC-Leu	ATG-Met
Ejs	ACC-Thr	ATC-Met	GCA-Ala	GGT-Gly	CTT-Leu	ATG-Met	CTT-Leu	GTC-Val	ACC-Thr	GCT-Ala	CGA-Arg	TTA-Leu	GTG-Val
En	GTA-Val	ATC-Met	GCA-Ala	AGT-Ser	ATT-Ile	CTG-Leu	CTT-Leu	GTC-Val	ACC-Thr	GTT-Val	CGA-Arg	ATC-Ile	ATG-Met
Ez	GTT-Val	ACC-Thr	ATA-Met	GGT-Gly	ATT-Ile	ATG-Met	CTT-Leu	CTC-Leu	GCC-Ala	GTT-Val	CGA-Arg	GTC-Val	CTC-Leu
Et	GTC-Val	ACC-Thr	GCA-Ala	AGT-Ser	CTT-Leu	TTA-Leu	CTT-Leu	CTC-Leu	ACC-Thr	GCT-Ala	CGA-Arg	GTT-Val	TTA-Leu

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 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 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

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 macrolepidia: accession number DQ536430, <https://identifiers.org/ncbiprotein:DQ536430>.

Percina crypta: accession number KY965073, <https://identifiers.org/ncbiprotein:KY965073>.

Etheostoma chuckwachatte: accession number KY965071, <https://identifiers.org/ncbiprotein:KY965071>.

Etheostoma caeruleum: accession number KY660678, <https://identifiers.org/ncbiprotein:KY660678>.

Etheostoma radiosum: accession number AY341348, <https://identifiers.org/ncbiprotein:AY341348>.

Etheostoma okaloosae: accession number KY747492, <https://identifiers.org/ncbiprotein:KY747492>.

Etheostoma spectabile: accession number MK243404, <https://identifiers.org/ncbiprotein:MK243404>.

Etheostoma jessiae: accession number KY965072, <https://identifiers.org/ncbiprotein:KY965072>.

Etheostoma nigrum: accession number KT289926, <https://identifiers.org/ncbiprotein:KT289926>.

Etheostoma zonale: accession number AP005994, <https://identifiers.org/ncbiprotein:AP005994>.

Etheostoma tallapoosae: accession number KY952221, <https://identifiers.org/ncbiprotein:KY952221>.

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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Open Peer Review

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Version 2

Reviewer Report 04 December 2019

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Dongsheng Zhang

Shanghai Ocean University, Shanghai, China

The authors tried to detect positive selection in mtDNA coding proteins within 11 species of darters, using a selection model, MEME and FEL method. they found some positive selection signals with six genes, and they detected one common site in CYTB using different methods.

Here are a couple of pieces of suggestion for the paper:

1. This paper should be able to present a better biological question and answers to it, such as evolutionary constraints acted on the mtDNA of darters with different habitats. If they could take a distinct environmental factor as evolutionary constraint and found their effects on the evolution of the darters, it will make this paper more biologically meaningful.
2. To test adaptive evolution, I suggest the author try models in codeml package in PAML, so they could test if the evolutionary rate differs for different species/lineages, and which gene(s) are under positive selection in the lineages of interest¹. Furthermore, they could use RELAX to test if the branches of interest is under relaxed selection or intensified selection².
3. They could project the sites under positive selection to the 3D models of the proteins, which will show the effect of the evolution events at protein level. Furthermore, if they could link the substitution of amino acids to the biological function of the proteins, the paper will be more attractive.

I think the authors still need to do a lot of work.

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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?

Yes

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

Partly

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

Yes

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.

Reviewer Report 14 November 2019

<https://doi.org/10.5256/f1000research.22996.r55422>

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Dusan Kordis

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

The authors have addressed some of my concerns.

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

No

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

No

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

No

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

No

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

No

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 confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 09 May 2019

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Dusan Kordis

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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.

References

1. da Fonseca RR, Johnson WE, O'Brien SJ, Ramos MJ, et al.: The adaptive evolution of the mammalian mitochondrial genome. *BMC Genomics*. 2008; **9**: 119 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Castoe TA, Jiang ZJ, Gu W, Wang ZO, et al.: Adaptive evolution and functional redesign of core metabolic proteins in snakes. *PLoS One*. 2008; **3** (5): e2201 [PubMed Abstract](#) | [Publisher Full Text](#)
3. Xia X, Kumar S: Codon-based detection of positive selection can be biased by heterogeneous distribution of polar amino acids along protein sequences. *Comput Syst Bioinformatics Conf*. 2006. 335-40 [PubMed Abstract](#)

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.

References:

Romero, P.A. and Arnold, F.H. 2009. Exploring protein fitness landscapes by directed evolution. *Nature Reviews. Molecular Cell Biology* 10(12), pp. 866–876.

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Spiller, B., Gershenson, A., Arnold, F.H. and Stevens, R.C. 1999. A structural view of evolutionary divergence. *Proceedings of the National Academy of Sciences of the United States of America* 96(22), pp. 12305–12310.

Competing Interests: No competing interests were disclosed.

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