CONVERGENT EVOLUTION OF CONSERVED MITOCHONDRIAL PATHWAYS UNDERLIES REPEATED ADAPTATION TO EXTREME ENVIRONMENTS

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Abstract

Extreme environments test the limits of life. Still, some organisms thrive in harsh conditions, begging the question whether the repeated colonization of extreme environments is facilitated by predictable and repeatable evolutionary innovations. We identified the mechanistic basis underlying convergent evolution of tolerance to hydrogen sulfide (H_2S)—a potent toxicant that impairs mitochondrial function—across evolutionarily independent lineages of a fish (*Poecilia mexicana*, Poeciliidae) from H_2S-rich freshwater springs. We found that mitochondrial function is maintained in the presence of H_2S in sulfide spring *P. mexicana*, but not ancestral lineages in adjacent nonsulfidic habitats, due to convergent adaptations in both the primary toxicity target and a major detoxification enzyme. Additionally, we show that H_2S tolerance in 10 independent lineages of sulfide spring fishes across multiple genera of Poeciliidae is mediated by convergent modification and expression changes of genes associated with H_2S toxicity and detoxification. Our results demonstrate that the repeated modification of highly conserved physiological pathways associated with essential mitochondrial processes enabled the colonization of novel environments.

37 Stephen J. Gould was a fierce proponent of the importance of contingency in evolution, famously
38 quipping that replaying the "tape of life" would lead to different outcomes every time (*1*).
39 Mitochondrial genomes were historically thought to be a prime example of such contingency
40 evolution, because alternative genetic variants were assumed to be selectively neutral (*2*). This
41 paradigm has been shifting, with mounting evidence that mitochondria—and genes encoded in the
42 mitochondrial genome—play an important role in adaptation, especially in the context of
43 physiochemical stress (*3*). However, it often remains unclear how genetic variation in mitochondrial
44 genomes and nuclear genes that contribute to mitochondrial function translates to variation in
45 physiological and organismal function. Furthermore, it is not known whether exposure to similar
46 selective regimes may cause convergent modifications of mitochondrial genomes and emergent
47 biochemical and physiological functions in evolutionarily independent lineages. Extreme
48 environments that represent novel ecological niches are natural experiments to address questions
49 about mechanisms underlying mitochondrial adaptations and illuminate the predictability of adaptive
50 evolution of mitochondria. Among the most extreme freshwater ecosystems are springs with high
51 levels of hydrogen sulfide (H_2S), a potent respiratory toxicant lethal to metazoans due to its
52 inhibition of mitochondrial ATP production (*4*). Multiple lineages of livebearing fishes (Poeciliidae)
53 have colonized H_2S-rich springs throughout the Americas and independently evolved tolerance to
54 sustained H_2S concentrations orders of magnitudes higher than those encountered by ancestral
55 lineages in nonsulfidic habitats (*5*). Here, we identify the mechanistic basis of increased H_2S
56 tolerance—an evolutionary innovation that facilitated the independent colonization of extreme
57 environments—and ask if the underlying mechanisms have evolved in convergence in disparate
58 lineages of livebearing fishes.
59 H_2S toxicity and detoxification are associated with highly conserved physiological pathways
60 in mitochondria (Figure 1A) (*6, 7*), providing *a priori* predictions about molecular mechanisms

61 underlying adaptation to this strong source of selection. Toxic effects of H_2S result from binding to
62 and inhibition of cytochrome c oxidase (COX) in the oxidative phosphorylation (OxPhos) pathway
63 (*8*). Animal cells can also detoxify low concentrations of endogenously produced H_2S via the
64 mitochondrial sulfide:quinone oxidoreductase (SQR) pathway, which is linked to OxPhos (*9*). We
65 have previously shown that genes associated with both pathways are under divergent selection and
66 differentially expressed between fish populations in sulfidic and nonsulfidic habitats (*5*). These
67 include genes encoding subunits of the direct toxicity target (COX) and the enzyme mediating the
68 first step of detoxification (SQR) (*5*). Tolerance to H_2S may therefore be mediated by resistance
69 (modification of toxicity targets that reduce the negative impact of H_2S), regulation (modification of
70 physiological pathways that maintain H_2S homeostasis), or both (*4*).

71 We used a series of *in vivo* and *in vitro* assays to identify the functional consequences of
72 modifications to the OxPhos and SQR pathways in evolutionarily independent population pairs of
73 *P. mexicana* from adjacent sulfidic and nonsulfidic habitats (Figure S1), including analyses of the
74 activity of relevant proteins and the physiological function of mitochondria and whole organisms. If
75 resistance was the primary mechanism of tolerance, we predict that COX function is maintained in
76 the presence of H_2S in fish from sulfidic populations, but not those from nonsulfidic populations.
77 Quantification of COX function indicated that enzyme activity generally declined with increasing
78 H_2S concentrations (Figure 1B). However, inhibition of COX by H_2S was reduced for two *P.*
79 *mexicana* populations from sulfidic habitats (Puy and Pich), which maintained significant COX
80 activity even at the highest H_2S concentrations. Consequently, resistance may contribute to H_2S
81 tolerance in some populations, but cannot explain the repeated evolution of H_2S tolerance by itself,
82 because COX activity in one H_2S-tolerant population (Tac) declined just as in nonsulfidic
83 populations (Figure 1B).

84 We also tested whether tolerant and intolerant populations differ in their ability to detoxify
85 H_2S by conducting enzyme activity assays of SQR. SQR activity was significantly higher in
86 mitochondria from sulfidic populations at intermediate and high H_2S concentrations (Figure 1C),
87 likely helping fish from sulfidic habitats to maintain H_2S homeostasis during environmental
88 exposure. To test this prediction *in vivo*, we used a novel mitochondria-specific H_2S-probe (MitoA)
89 that allows for the monitoring of relative H_2S levels inside the mitochondria of living organisms (*10*).
90 We measured mitochondrial H_2S concentrations in this manner using laboratory-reared fish that
91 were exposed to varying levels of H_2S. Mitochondrial H_2S concentrations in mitochondria isolated
92 from livers (Figure 1D) and other organs (Figure S2) of fish from nonsulfidic habitats increased
93 above control levels at all exposure concentrations. In contrast, mitochondrial H_2S concentrations in
94 isolates of fish from sulfidic populations did not usually exceed control levels and remained lower
95 than levels in fish from nonsulfidic habitats. Together, these results indicate that populations of *P.*
96 *mexicana* from sulfidic habitats can detoxify H_2S at higher rates and thus regulate mitochondrial H_2S
97 upon environmental exposure.
98 Modification of the OxPhos and SQR pathways in *P. mexicana* suggests that mitochondrial
99 adaptation is key to the evolution of H_2S tolerance. Therefore, mitochondrial function of fish from
100 sulfidic habitats should be maintained upon exposure to H_2S. We tested this hypothesis by
101 quantifying different aspects of mitochondrial function (basal respiration, maximal respiration, and
102 spare respiratory capacity) along a gradient of H_2S concentrations using an *ex vivo* coupling assay. As
103 expected, all aspects of mitochondrial function generally declined with increasing H_2S (Figures 1E,
104 S3-S5). Comparison of mitochondrial function between adjacent populations in sulfidic and
105 nonsulfidic habitats indicated no differences in basal respiration (Figure S3). However, individuals
106 from sulfidic populations were able to maintain maximal respiration and spare respiratory capacity at
107 higher levels compared to individuals from nonsulfidic habitats of the same river drainage (Figure

108 1E), even though the magnitude of difference and the shape of response curves varied (Figures S4-
109 S5). Overall, our findings indicate that mitochondria of H_2S-tolerant individuals continue to produce
110 ATP in the presence of an inhibitor that reduces mitochondrial function in ancestral lineages.

111 The independent evolution of H_2S tolerance in *P. mexicana* by convergent modifications in
112 pathways involved in toxicity and detoxification begs questions about the origin of adaptive alleles
113 (*11*). At microevolutionary scales, convergence may be a consequence of the repeated assembly of
114 related alleles into different genomic backgrounds, either through selection on standing genetic
115 variation or introgression (*12, 13*). However, the epitome of convergent evolution is arguably the
116 independent origin of adaptive mutations at the same locus that lead to consistent functional
117 outcomes (*14, 15*). To identify convergence at a genomic level, we re-sequenced whole genomes of
118 multiple *P. mexicana* individuals from sulfidic and nonsulfidic habitats. Analyzing phylogenetic
119 relationship among *P. mexicana* populations (with *P. reticulata* as an outgroup) using 13,390,303 SNPs
120 distributed across the genome confirmed three independent colonization events of sulfide springs
121 and distinct evolutionary trajectories for sulfide spring populations in different drainages (Figure
122 2A). If adaptive alleles arose separately through *de novo* mutation in each sulfide spring population,
123 we would expect that putative adaptive alleles mirror these relationships, as previously documented
124 for H_2S-resistant alleles in mitochondrial *COX* subunits (*16*). However, patterns of divergence
125 (Figure S6) and local ancestry were highly variable across the genome. Classifying local patterns of
126 genetic similarity using a Hidden Markov Model and a Self Organising Map (*17*) allowed us to
127 identify genomic regions in which ancestry patterns deviate from the genome-wide consensus,
128 including multiple regions with a strong signal of clustering by ecotype (sulfidic *vs.* nonsulfidic
129 populations). Such clustering by ecotype occurred in <1 % of the genome (Figure S7), but included
130 genomic regions encoding genes associated with H_2S detoxification (e.g., ETHE1 and SQR; Figure
131 2B, Table S14). Clustering by ecotype indicates a monophyletic origin of putatively adaptive alleles

132 that are shared across independent lineages of sulfide spring *P. mexicana* as a consequence of
133 selection on standing genetic variation or introgression. Consequently, multiple mechanisms played a
134 role in the convergent evolution of H_2S-tolerance in *P. mexicana*.

135 While selection on standing genetic variation and introgression can explain convergent
136 evolution at microevolutionary scales, adaptive alleles are unlikely to be shared among lineages at
137 macroevolutionary scales due to high phylogenetic and geographic distances separating gene pools
138 (*18*). Absence of convergence in molecular mechanisms at broader phylogenetic scales might
139 indicate the importance of contingency in evolution, as postulated by Gould. In contrast, the
140 presence of convergence would indicate that fundamental constraints limit the number of solutions
141 for a functional problem (*19*). We used phylogenetic comparative analyses of gene expression and
142 analyses of molecular evolution to detect patterns of molecular convergence in 10 lineages of sulfide
143 spring poeciliids and ancestors in nonsulfidic habitats (Figure S1). This included members of five
144 genera that span over 40 million years of divergence and occur in different biogeographic contexts
145 (Figure S1). We found evidence for convergence in both gene expression and sequence evolution.
146 Variation in overall gene expression was strongly influenced by phylogenetic relationships (Figure
147 3A). However, 186 genes exhibited significant evidence for convergent expression shifts in sulfide
148 spring fishes (Figure 3B, Table S16), segregating lineages based on habitat type of origin, irrespective
149 of phylogenetic relationships (Figure 3C). Functional annotation indicated that genes with
150 convergent expression shifts were enriched for biological processes associated with H_2S
151 detoxification (SQR pathway, Figure 3D), the processing of sulfur compounds, and H_2S toxicity
152 targets in OxPhos (Figure S8, Table S17). We also identified 11 genes with elevated nonsynonymous
153 to synonymous substitution rates across the phylogeny, including three mitochondrial genes that
154 encode subunits of H_2S's toxicity target (*COX1* and C*OX3*) and OxPhos complex III (*CYTB*; Table
155 S18). Most amino acid substitutions in *COX1* and *COX3* occurred in a lineage-specific fashion, but

156 convergent substitutions across clades occurred at six codons in *COX1* and two codons in *COX3*
157 (Figure 4).

158 Colonization of novel niches with extreme environmental conditions in poeciliids is the
159 result of repeated and predicted modifications of the same physiological pathways, genes, and—in
160 some instances—codons associated with mitochondrial function. This convergence at multiple
161 levels of biological organization is likely a consequence of constraint, because the explicit
162 biochemical and physiological consequences of H_2S severely limit the ways organisms can cope with
163 its toxicity (*19*). Due to these constraints, molecular convergence is not only evident at
164 microevolutionary scales, where selection can repeatedly assemble related alleles into different
165 genomic backgrounds, but also at macroevolutionary scales including lineages separated by over 40
166 million years of evolution. Evolutionary novelty can consequently arise through the convergent
167 modification of the most conserved physiological pathways, underscoring the long overlooked role
168 of mitochondria in adaptive evolution (*3*).

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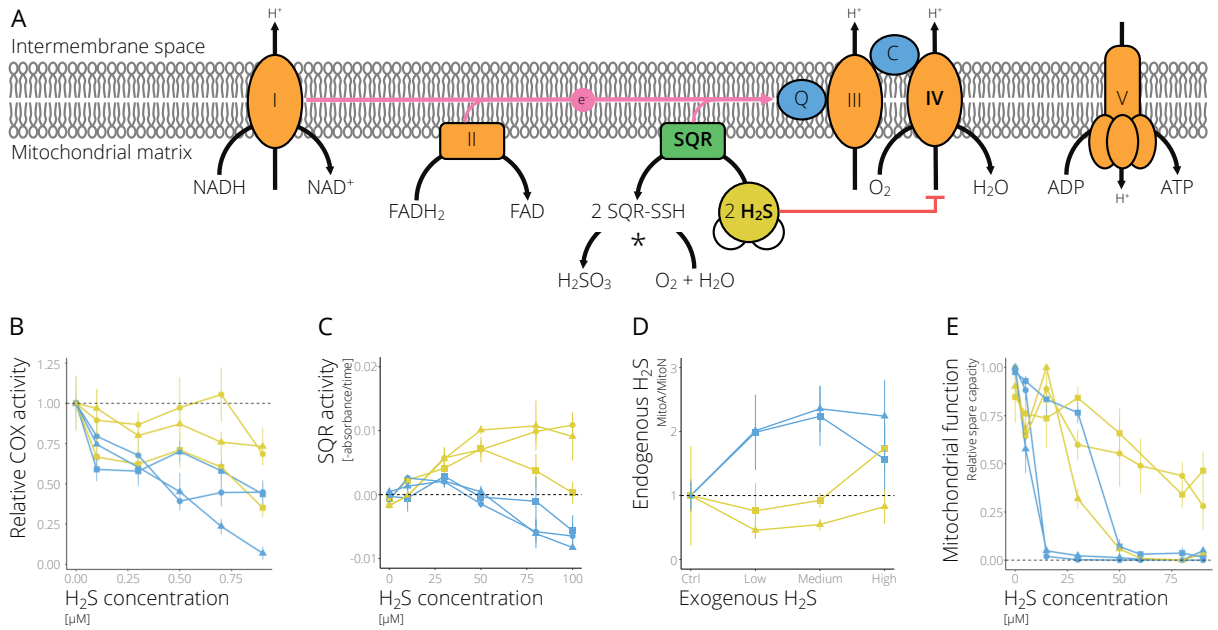

Figure 1.A. Physiological pathways associated with H_2S toxicity and detoxification are located in the inner mitochondrial membrane. H_2S inhibits OxPhos (orange enzymes) by binding to COX (Complex IV). H_2S can be detoxified through SQR (green enzyme) and additional enzymes (indicated by asterisks). **B.** Relative activity of COX upon H_2S exposure, which was primarily explained by an interaction between habitat type of origin and ambient H_2S concentration (Tables S2-S3). **C.** Activity of SQR as a function of H_2S concentration, which was explained by an interaction between habitat type of origin and H_2S concentration (Tables S4-S5). **D.** Relative change in mitochondrial H_2S concentrations in the liver of live fish exposed to different levels of environmental H_2S. Variation in mitochondrial H_2S levels were explained by habitat type of origin and exogenous H_2S concentration (Tables S6-S7). **E.** Relative spare respiratory capacity of isolated liver mitochondria at different levels of H_2S. The interaction between habitat type of origin and drainage of origin best explained variation in spare respiratory capacity (Tables S11-S12). For all graphs, yellow colors denote *P. mexicana* from H_2S-rich habitats, blue from nonsulfidic habitats. Symbols stand for populations from different river drainages (■: Tac; ▲: Puy; ●: Pich; see Figure S1).

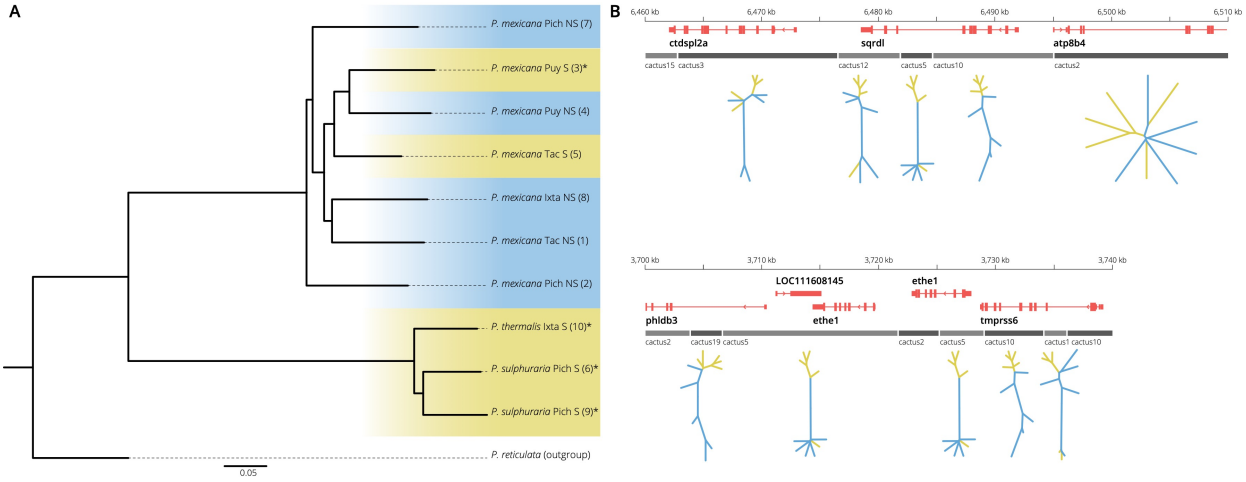

Figure 2.A. Phylogeny of different population in the *P. mexicana* species complex (with *P. reticulata* as an outgroup) based on genome-wide SNPs. Colors indicate nonsulfidic (blue) vs. sulfidic (yellow) lineages. **B.** Local ancestry patterns around two key genes involved in H_2S detoxification, *SQR* and *ETHE1*. Gray bars represent the local ancestry pattern (cactus) associated with each region. Unrooted trees represent local ancestry relationships, with sulfidic lineages colored in yellow and nonsulfidic lineages in blue. Cacti 10 and 19 show clear clustering by ecotype. In cacti 1, 5, and 12, four of five sulfidic individuals cluster together.

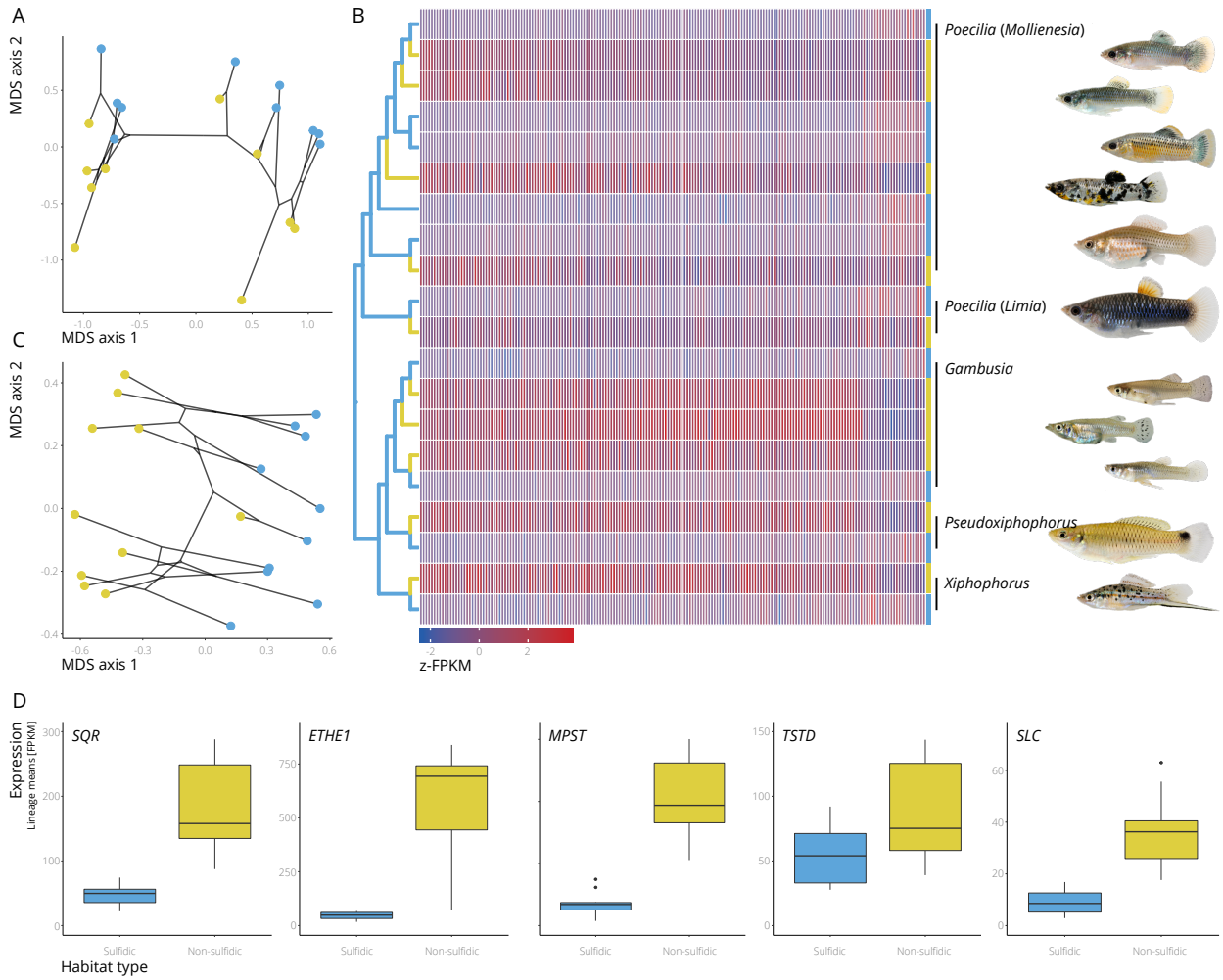

Figure 3.A. Multidimensional scaling (MDS) plot of overall gene expression patterns across 20 lineages of poeciliid fishes. Black lines represent phylogenetic relationships among lineages; color represents habitat type of origin (yellow: sulfidic; blue: nonsulfidic). **B.** Expression variation of 186 genes with evidence for convergent expression shifts (z-transformed). Colors represent expression levels as indicated by the scale. The cladogram shows the phylogenetic relationship among lineages. Pictures on the side are examples of sulfide spring fishes (from top to bottom): *P. mexicana* (Tac), *P. mexicana* (Puy), *P. sulphuraria* (2), *P. latipinna*, *L. sulphurophila*, *G. sexradiata*, *G. eurystoma*, *G. holbrooki*, *P. bimaculatus*, *X. hellerii*. **C.** MDS plot of the expression of 186 genes with evidence for convergent expression shifts. **D.** Boxplot with mean expression levels of different components of the SQR pathway across lineages from sulfidic (yellow) and nonsulfidic (blue) habitats.

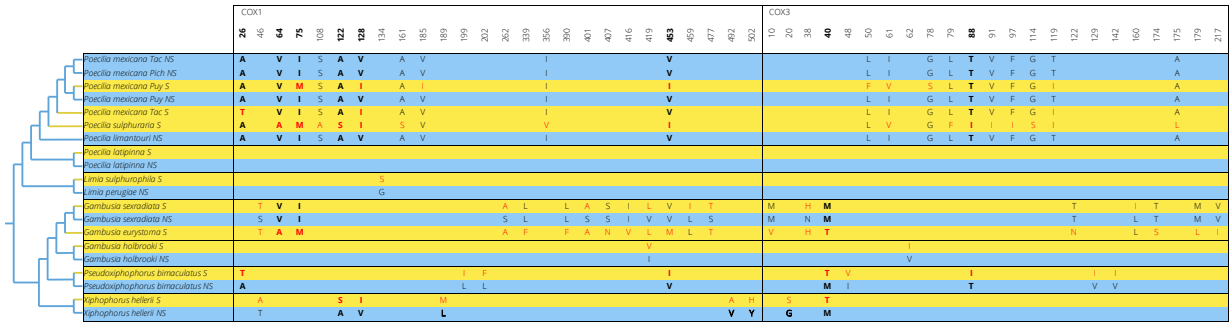

Figure 4. Amino acid differences in *COX1* and *COX3* between lineages from sulfidic (yellow) and nonsulfidic (blue) habitats. Derived amino acids are shown in red. Bold letters indicate codons with convergent amino acid substitutions in different clades (separated by black horizontal lines) of sulfide spring fishes.