

1 Disentangling positive vs. relaxed selection in animal mitochondrial genomes

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15

16 **Abstract**

17

18 Disentangling different types of selection is a common goal in molecular evolution. Elevated  
19  $d_N/d_S$  ratios (the ratio of nonsynonymous to synonymous substitution rates) in focal lineages  
20 are often interpreted as signs of positive selection. Paradoxically, relaxed purifying selection  
21 can also result in elevated  $d_N/d_S$  ratios, but tests to distinguish these two causes are seldomly  
22 implemented. Here, we reevaluated seven case studies describing elevated  $d_N/d_S$  ratios in  
23 animal mtDNA and their accompanying hypotheses regarding selection. They included flightless  
24 vs. flighted lineages in birds, bats, and insects, and physiological adaptations in snakes, two  
25 groups of electric fishes, and primates. We found that elevated  $d_N/d_S$  ratios were often not  
26 caused by the predicted mechanism, and we sometimes found strong support for the opposite  
27 mechanism. We discuss reasons why energetic hypotheses may be confounded by other  
28 selective forces acting on mtDNA and caution against overinterpreting molecular “spandrels”,  
29 including elevated  $d_N/d_S$  ratios.

30

31 Keywords: organelle, RELAX, mitogenome, metabolism, adaptation, brain-energy hypothesis,  
32 flightless

### 33 Introduction

34 Detecting different patterns of selection using sequence data is a key goal of molecular  
35 evolution. A popular, simple tool in such studies is the  $d_N/d_S$  ratio: the ratio of nonsynonymous  
36 to synonymous substitution rates. An abundance of nonsynonymous changes ( $d_N/d_S > 1$ ) may  
37 indicate positive selection for a gene to undergo adaptive amino acid replacement, while  $d_N/d_S$   
38 ratios near 1 suggest neutral evolution, and  $d_N/d_S$  ratios  $< 1$  indicate purifying selection (Hughes  
39 and Nei 1989). Researchers often employ “branch models” to estimate  $d_N/d_S$  ratios across a  
40 phylogeny (Yang and Nielsen 1998), with elevated  $d_N/d_S$  ratios in focal lineages suggesting  
41 intensified positive selection. Paradoxically, elevated  $d_N/d_S$  ratios can also indicate relaxed  
42 purifying selection owing to increased amino acid changes being tolerated more in focal  
43 lineages. However, studies rarely explicitly disentangle relaxed vs. positive selection as causes  
44 of elevated  $d_N/d_S$  ratios.

45 Specifically, elevated  $d_N/d_S$  ratios in mitochondrial DNA (mtDNA) are often implicated as  
46 evidence for positive selection on energetics. Despite being originally assumed to be a strictly  
47 neutral marker (Ballard and Kreitman 1995; Brown et al. 1979; Lynch 1996), mtDNA variation  
48 has been convincingly linked to adaptation across different types of environments (Ballard and  
49 Melvin 2010; Ballard and Whitlock 2004; Camus et al. 2017; Chevion and Brumfield 2009;  
50 Dobler et al. 2014; Greenway et al. 2020; Hill 2019; James and Ballard 2003), with 26% of  
51 nonsynonymous substitutions in animal mtDNA likely fixed by adaptive evolution (James et al.  
52 2016). Given the myriad roles mitochondria play in cellular metabolism, mtDNA variation may  
53 be widely important in animal adaptive evolution (Havird et al. 2019b; Hill et al. 2019).  
54 Therefore, it is tempting to speculate that signs of positive selection on mtDNA may be found in  
55 any animal lineage that occupies an interesting ecological or metabolic niche. Specific  
56 hypotheses can be readily tested due to the availability of complete metazoan mtDNA  
57 sequences (Fig. 1) and tools for extracting complete mtDNAs from next-generation sequencing  
58 datasets (Al-Nakeeb et al. 2017; Allio et al. 2020; Guo et al. 2013; Nachtigall et al. 2021).  
59 Examples of recently tested hypotheses include positive selection on mtDNA for low  
60 echolocation frequencies in bats (Zhang et al. 2021) and environmental adaptation in gentoo  
61 penguins (Noll et al. 2022).

62 Studies of selection on mtDNA offer a useful example to demonstrate the importance of  
63 disentangling positive vs. relaxed selection when interpreting  $d_N/d_S$  results. Relaxed selection  
64 on mtDNA (relative to control lineages) is seldom explicitly explored given the core functions of  
65 mt genes. However, relatively relaxed selection on mtDNA has been convincingly demonstrated  
66 in eusocial shrimps (Chak et al. 2020) and bivalves with doubly-uniparental inheritance of  
67 mtDNA (Maeda et al. 2021). These studies used tests that specifically discriminate positive from  
68 relaxed selection and found consistent, global signatures of relaxed selection along with  
69 elevated  $d_N/d_S$  ratios.

70 Here, we reanalyzed seven case studies where positive or relaxed selection was  
71 hypothesized based on elevated  $d_N/d_S$  ratios from mtDNA datasets. Three cases concerned the  
72 evolution/loss of flight and four investigated metabolic innovations. We expanded taxon  
73 sampling, calculated branch-specific  $d_N/d_S$  ratios, and used RELAX (Wertheim et al. 2014) to  
74 distinguish between positive and relaxed selection on complete mitogenomes. While some  
75 conclusions of the original studies may remain valid, our analyses suggest that assuming  
76 elevated  $d_N/d_S$  ratios fit a particular narrative without explicitly testing for their causes may lead  
77 to erroneous, spandrel-esque conclusions (Gould et al. 1979).

78

## 79 **Materials and Methods**

### 80 *Positive and relaxed selection in flighted and flightless lineages*

81 The first three case studies we reexamined explored the general hypothesis that mtDNA  
82 is under positive selection in flighted compared to flightless lineages because flight is  
83 energetically intense. First, mtDNA in bats was hypothesized to be under positive selection  
84 compared to flightless mammals (Shen et al. 2010). We therefore compared 77 bats to 58  
85 flightless mammals (including insectivores, carnivores, cetaceans, and ungulates, similar to  
86 Shen et al. (2010). For the second and third case studies, mtDNA in flightless birds and insects  
87 was hypothesized to be under relatively relaxed selection compared to flighted lineages  
88 (Mitterboeck and Adamowicz 2013; Shen et al. 2009). We therefore compared 49 flightless  
89 birds to 47 closely related flighted birds and 50 flightless insects to 56 closely-related flighted  
90 insects. For birds, species were classified as flighted if they were at least weakly flighted, so

91 tinamous were classified as flighted, while emus and kiwis are considered flightless. Because  
92 flight can be more plastic in insects, we categorized ambiguous species as flighted if they  
93 produced flighted reproductive females. This is because mitochondria are transmitted only by  
94 females and flight can only be selected for via mtDNA changes in females (e.g., Havird et al.  
95 2019a).

96

#### 97 *Positive selection for metabolic innovations*

98         The four other case studies hypothesized positive selection on mtDNA in lineages with  
99 energetic innovations. First, in snakes compared to lizards, owing to physiological redesign  
100 associated with lung volume reduction, consumption of infrequent/large meals, and venom  
101 production (Castoe et al. 2008). We therefore compared 21 snakes to 31 closely related lizards.  
102 The next two case studies hypothesized positive selection in two independent origins of electric  
103 fishes due to their ability to generate electric fields for communication and prey detection  
104 (Elbassiouny et al. 2020). Therefore, for South American gymnotiform electric fishes we  
105 compared 44 gymnotiforms to 43 closely related characiforms and for the African mormyroid  
106 electric fishes we compared 39 mormyroids to 79 closely related osteoglossiforms/clupeiforms.  
107 Finally, in the last case study we reexamined the “brain-energy” hypothesis, which states that  
108 accelerated evolution of mtDNA in primates reflects adaptive evolution for enhanced brain  
109 function (Goldberg et al. 2003; Grossman et al. 2004; Osada and Akashi 2012). We therefore  
110 compared 10 primates to 20 closely-related rodents, ungulates, and carnivores. We note that in  
111 the bats, snakes, electric fishes, and the primate cases, there is only a single, monophyletic  
112 lineage of interest, limiting the phylogenetic power of the analyses compared to the bird and  
113 insect cases, where many independent flightless lineages were examined.

114

#### 115 *Sequence curation*

116         For each case study we first searched NCBI’s organelle genome database for complete  
117 mtDNA sequences from relevant species. In some datasets with limited availability of complete  
118 mitogenomes, we used other resources, including the EFISH Genomics 2.0 database for electric  
119 fishes (<https://efishgenomics.integrativebiology.msu.edu/data/>) and Campagna et al. (2019) for

120 flightless birds, where transcriptomes/genomes were searched for mt genes using BLAST  
121 (Altschul et al. 1997). For electric fishes and flightless birds, we also *de novo* assembled and  
122 annotated complete (or nearly complete) mitogenomes from publicly available Illumina  
123 sequencing data. FASTQ files were downloaded from NCBI's Sequence Read Archive (SRA) and  
124 mitogenomes were assembled using MitoFinder v1.4 with default settings (Allio et al. 2020).  
125 We therefore report new mitogenomes from six birds, 26 gymnotiforms, and nine mormyroids  
126 (GenBank accessions: BK061684-BK061730, Table S1).

127

### 128 *Disentangling positive vs. relaxed selection on mtDNA*

129 We first extracted nucleotide sequences from the 13 mitochondrial protein-coding  
130 genes in each mitogenome. For each gene we translated and aligned the resulting amino acid  
131 sequences using the proper translation table (either the vertebrate or invertebrate  
132 mitochondrial genetic code) for each of the seven datasets outlined above, using Muscle (Edgar  
133 2004) as implemented in MEGA X (Kumar et al. 2018). Resulting alignments were manually  
134 corrected by eye and both amino acid and nucleotide sequences were exported as fasta files.  
135 Alignments were concatenated to make a "mitogenome" dataset representing all 13 genes.  
136 Amino acid sequences from the mitogenome dataset were used to perform phylogenetic  
137 reconstruction under maximum likelihood in RAxML v8.2.12 with the gamma WAG model of  
138 amino acid substitution and 100 rapid bootstrap replicates (`-f a -# 100 -m PROTGAMMAWAG`)  
139 (Stamatakis 2014; Stamatakis et al. 2008). Resulting topologies were rooted according to the  
140 original publications referenced above to guide selection analyses. For flightless insects we used  
141 a constrained topology at the level of orders based on Misof et al. (2014).

142 We first used branch-specific models in PAML v4.8 (Yang 2007) to fit two different  $d_N/d_S$   
143 ratios on phylogenetic branches in each dataset ("model = 2"): one for the focal/test lineage(s)  
144 (red branches in Figs. 2-3, including terminal and consensus internal branches) and one for  
145 reference lineages. Internal branches with descendants in both character states (e.g., flighted  
146 and flightless) were coded with the ancestral condition by default (e.g., flighted in insects). We  
147 compared the resulting likelihood scores from this model to one where all branches were fit

148 with a single  $d_N/d_S$  ratio (“model = 0”) using a likelihood ratio test. This was performed for each  
149 gene individually and the mitogenome dataset in each case study.

150 To distinguish whether positive vs. relaxed selection in focal lineages, we performed  
151 analyses using RELAX (Wertheim et al. 2014), both locally within the HyPhy package (Pond et al.  
152 2005) and on the datamonkey webserver (Weaver et al. 2018). Phylogenetic branches were  
153 coded as either “test” or “reference” as above (with reference indicating the ancestral  
154 condition). Briefly, RELAX compares the distribution of  $d_N/d_S$  ratios across sites in test vs.  
155 reference branches, summarized by a  $k$  parameter, with  $k < 1$  suggesting relatively relaxed  
156 selection and  $k > 1$  suggesting intensified/positive selection in test branches. The statistical  
157 significance of the  $k$  parameter is assessed by comparison to a model where a single  
158 distribution of  $d_N/d_S$  ratios is applied across all branches. We performed RELAX analyses on  
159 each gene individually and the mitogenome dataset in each case study.

160

#### 161 *Data availability*

162 All data analyzed here are publicly available and accession numbers are provided in  
163 Table S1. Relevant tree, alignment, and raw data files from this project are available via  
164 FigShare  
165 ([https://figshare.com/articles/dataset/Positive\\_vs\\_relaxed\\_mtDNA\\_selection/21277536](https://figshare.com/articles/dataset/Positive_vs_relaxed_mtDNA_selection/21277536); also  
166 happy to upload to Dryad).

167

## 168 **Results**

### 169 *Inconsistent selection on flighted vs. flightless lineages’ mtDNA*

170 As previously reported, we found increased  $d_N/d_S$  ratios in bats compared to flightless  
171 mammals (0.44 vs. 0.40 for the mitogenome,  $P < 0.001$ , Fig. 2B) (Shen et al. 2010). However,  
172 the signal was not consistent: 5 genes showed significantly elevated  $d_N/d_S$  ratios, *ATP6* had a  
173 significantly lower  $d_N/d_S$  ratio, and the rest showing non-significant differences. Contrary to the  
174 predicted hypothesis, RELAX analyses indicated this pattern was likely due to relaxed, not  
175 positive selection on bats ( $k = 0.93$  for the mitogenome,  $P < 0.001$ , Fig. 2C). No individual gene

176 showed the predicted pattern of an elevated  $d_N/d_S$  ratio associated with a signal of positive  
177 selection (Fig. 2).

178 For birds, flightless lineages did not have elevated  $d_N/d_S$  ratios as reported previously  
179 (Shen et al. 2009) (0.04 vs. 0.04 for the mitogenome,  $P = 0.717$ , Fig. 2B). RELAX analyses  
180 indicated a weak, but significant trend towards intensified, not relaxed, selection in flightless  
181 lineages ( $k = 1.04$  for the mitogenome,  $P = 0.034$ , Fig. 2C). However, only *ND2* showed a  
182 significant pattern of elevated  $d_N/d_S$  ratios in flightless taxa (0.06 vs. 0.05,  $P < 0.001$ ) consistent  
183 with relaxed selection ( $k = 0.91$ ,  $P = 0.043$ ).

184 Contrary to previous results (Mitterboeck and Adamowicz 2013), lower, not elevated,  
185  $d_N/d_S$  ratios were observed in flightless compared to flighted lineages (0.03 vs. 0.04 for the  
186 mitogenome,  $P < 0.001$ , Fig. 2B), which was fairly consistent across genes (Fig. 2B). RELAX  
187 analyses were largely statistically non-significant (Fig. 2C), although  $k$  values in general  
188 suggested relaxed selection on flightless lineages. Three individual genes (*ATP6*, *ATP8*, and  
189 *CYTB*) did show the expected patterns of elevated  $d_N/d_S$  ratios associated with signals of relaxed  
190 selection.

191

#### 192 *Relaxed and positive selection on mtDNA in lineages with energetic innovations*

193 For snakes, we confirmed previously reported elevated  $d_N/d_S$  ratios compared to lizards  
194 (0.07 vs. 0.06 for the mitogenome,  $P = 0.002$ , Fig. 3B) (Castoe et al. 2008), which was consistent  
195 across genes (Fig. 3B). As hypothesized, this was likely due to positive selection ( $k = 1.1$ ,  $P <$   
196  $0.001$  for the mitogenome, Fig. 3C). However, individual genes were inconsistent. The only two  
197 genes with  $k$  values significantly greater than one (*ND1* and *ND4*) did not have significantly  
198 elevated  $d_N/d_S$  ratios. Three individual genes with significantly elevated  $d_N/d_S$  ratios showed  $k$   
199 values significantly less than one (*ATP6*, *ND5*, and *ND5*), suggesting relaxed, not positive  
200 selection on these genes.

201 For both gymnotiform and mormyroid electric fishes, there was a clear and consistent  
202 trend of elevated  $d_N/d_S$  ratios in electric taxa compared to closely related non-electric fishes,  
203 both in the mitogenome (difference in  $d_N/d_S$  ratios of 0.004 – 0.008,  $P < 0.001$  for mormyroids,  
204  $P = 0.182$  for gymnotiforms, Fig. 3B) and in individual mitochondrial genes (Fig. 3B), as

205 previously reported (Elbassiouny et al. 2020). However, this was not consistent with positive  
206 selection via the RELAX results. For mormyroids, there was a clear and consistent pattern of  
207 relaxed, not positive, selection ( $k = 0.83$ ,  $P < 0.001$  for the mitogenome, similar trends for  
208 individual genes, Fig. 3C). For gymnotiforms, there was a less clear pattern, with similar  
209 numbers of genes indicating relaxed and positive selection (the mitogenome had a small, but  
210 statistically significant effect of positive selection:  $k = 1.02$ ,  $P = 0.024$ , Fig. 3C).

211 As suggested previously, primates did show significantly elevated  $d_N/d_S$  ratios across the  
212 mitogenome (0.09 vs. 0.04,  $P < 0.001$ , Fig. 3B) and in nearly all individual genes (up to five-fold  
213 for *COX1*). Contradicting the brain-energy hypothesis (Grossman et al. 2004), there was a clear  
214 and consistent signal of relaxed, not positive-selection in primates, both in across the  
215 mitogenome ( $k = 0.51$ ,  $P < 0.001$ , Fig. 3C) and in nearly all individual genes (*ATP8* was the most  
216 extreme, being under 5.5 times less intense selection in primates).

217

## 218 Discussion

219 Our results suggest that some previous hypotheses may have been based on  
220 misinterpreting the underlying causes of elevated  $d_N/d_S$  ratios in mtDNA. We argue that  
221 distinguishing between relaxed and intensified positive selection is critical. Of the seven case  
222 studies we examined, none showed elevated  $d_N/d_S$  ratios that were consistently attributed to  
223 the predicted mechanism, and sometimes the opposite mechanism was convincingly  
224 supported. Other times, increased  $d_N/d_S$  ratios in focal lineages were not recovered as in the  
225 original analyses, possibly due to increased taxonomic sampling.

226 Three of the seven case studies we reexamined broadly hypothesize that the evolution  
227 of flight drives selection for energetic efficiency because flight is energetically expensive and  
228 requires highly efficient mitochondrial function. However, this hypothesis and others related to  
229 energetic efficiency in focal lineages, may not be falsifiable based on  $d_N/d_S$  ratios alone. Under  
230 this hypothesis, flighted lineages should show higher  $d_N/d_S$  ratios due to positive selection.  
231 However, elevated  $d_N/d_S$  ratios in *flightless* taxa due to relaxed selection would also support  
232 this hypothesis. Because finding increased  $d_N/d_S$  ratios in either flightless or flighted lineages



233 would support the overall hypothesis, it is difficult to falsify it. Only by distinguishing the cause  
234 of increased  $d_N/d_S$  ratios can the hypothesis be properly tested.

235         While positive selection on mitochondrial genes is an important part of environmental  
236 adaptation (Hill 2019), other forces also shape mtDNA evolution. Due to the uniparental  
237 inheritance, effective haploidy, and lack of recombination, mtDNA should be under less  
238 efficient selection overall than nuclear-encoded genes and have a smaller effective population  
239 size ( $N_e$ ) (Lynch 1996; Lynch and Blanchard 1998; Neiman and Taylor 2009). Differences in  $N_e$   
240 may also shape relative selection pressures on mtDNA among lineages (Bazin et al. 2006;  
241 Meiklejohn et al. 2007). For example, parasitic lineages often show accelerated mtDNA  
242 evolution compared to non-parasitic lineages, possibly owing to reduced  $N_e$  (Castro et al.  
243 2002; Jakovlić et al. 2021; Oliveira et al. 2008). Because flightless lineages may have lower  
244 dispersal abilities, they may also have lower  $N_e$  (Ikeda et al. 2012; McCulloch et al. 2009) and be  
245 under relatively relaxed selection, not because they lead a less energetic lifestyle. Many  
246 flightless taxa (especially among birds) are also associated with islands and inherently low  $N_e$   
247 (Woolfit and Bromham 2005). High  $d_N/d_S$  ratios in mormyroid electric fishes consistent with  
248 relaxed selection (Fig. 3) may stem from relatively low  $N_e$  compared to reference taxa such as  
249 Clupeiformes. Primates also had high  $d_N/d_S$  ratios due to relaxed selection, contrary to the  
250 brain-energy hypothesis (Goldberg et al. 2003; Grossman et al. 2004), but consistent with lower  
251  $N_e$  in primates compared to reference taxa such as rodents. Other factors independent of  
252 energetics also play confounding roles in mtDNA evolution, including longevity (Galtier et al.  
253 2009; Hua et al. 2015; Nabholz et al. 2008), generation time (Thomas et al. 2010), and other  
254 cytoplasmic endosymbionts (Hurst and Jiggins 2005).

255         Multiple factors may shape energetics in complementary or contradictory ways in  
256 specific lineages. For example, it has been argued that thermic habit (i.e., endothermy vs.  
257 ectothermy) and/or metabolic rate may predict patterns of mtDNA evolution (Rand 1993; Rand  
258 1994). Several of our flightless birds were penguins which may expend more energy on  
259 thermoregulation, while others were large, fast-running paleognathes that might have elevated  
260 metabolic rates (Fig. 2A). In these taxa, the lower energetic lifestyle stemming from flight loss is  
261 confounded with other high-energy adaptations, which may explain why overall  $d_N/d_S$  ratios

262 were similar between flighted and flightless taxa (Fig. 2B). Some studies do attempt to correct  
263 for confounding factors. For example, Mitterboeck and Adamowicz (2013) purposely excluded  
264 any cases of insect flight loss that were confounded with transitions in life history or population  
265 sizes. However, correcting for all confounding factors may be impractical.

266 Overall, we suggest a cautionary approach when interpreting molecular signatures like  
267  $d_N/d_S$  ratios that are easily calculable, especially as data become increasingly available (Fig. 1).  
268 Authors should be skeptical of adaptive “just-so stories” of genetic selection. Unfortunately,  
269 tools to explicitly disentangle relaxed vs. positive selection are limited (but see e.g., Crotty et al.  
270 2020). Other standard tools besides  $d_N/d_S$  ratios such as McDonald-Kreitman tests (McDonald  
271 and Kreitman 1991) can also have problems in certain contexts (e.g., with mtDNA, Meiklejohn  
272 et al. 2007). Selective hypotheses stemming from molecular evolution analyses should  
273 ultimately be evaluated through further functional experiments to avoid overinterpretation of  
274 molecular “spandrels” (Gould et al. 1979).

275

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279

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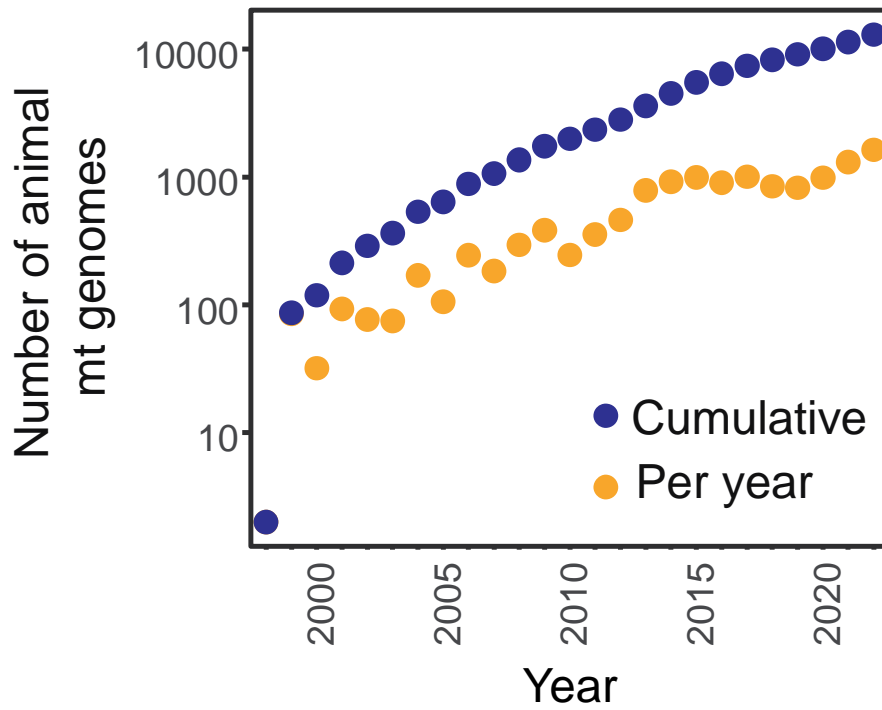
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442 **Figures and captions**



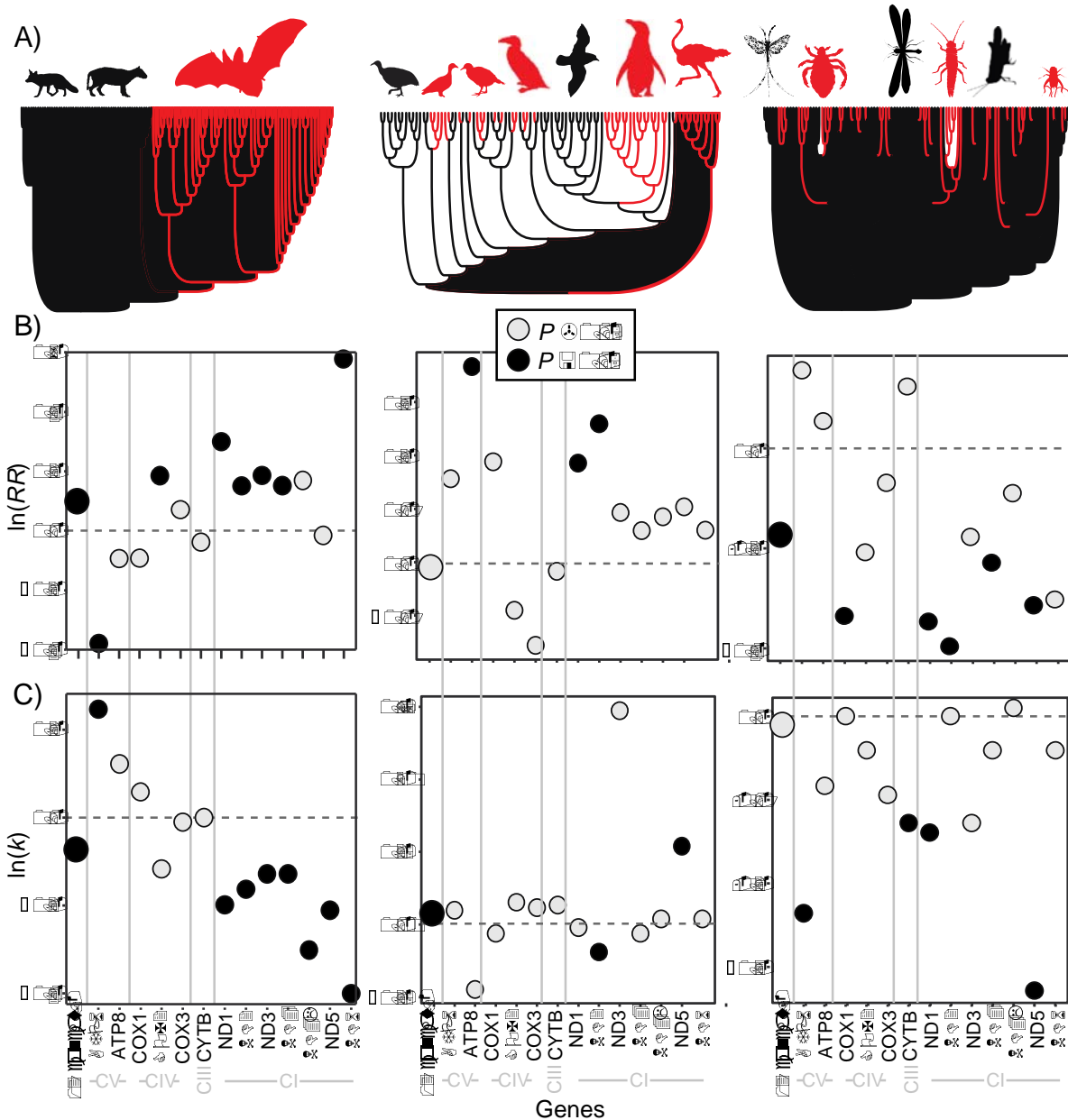
443

444 **Figure 1.** Number of complete animal mtDNA genomes available on NCBI's organelle database,

445 both per year and cumulative. Accessed on 5 August 2022.

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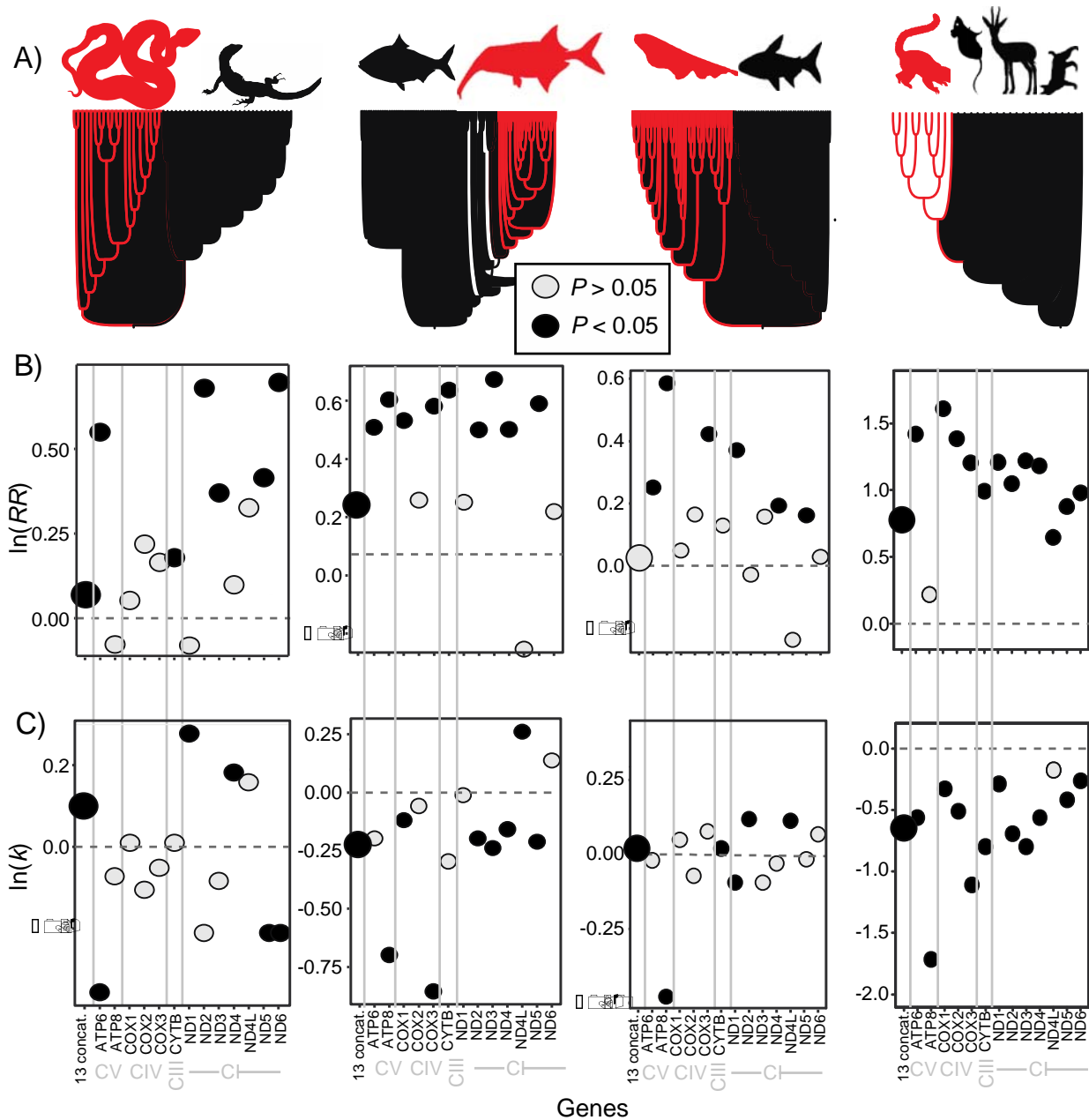
448

449 **Figure 2.** Selection on mitochondrial genes in flightless and flighted lineages. A) Topology  
 450 showing the number and distribution of lineages (in red; from left to right: bats, flightless birds,  
 451 and flightless insects) and reference lineages (in black). B)  $d_N/d_S$  ratios in focal lineages  
 452 compared to reference taxa. Data are presented as the natural log of the response ratio, with  
 453 numbers above zero indicating higher  $d_N/d_S$  ratios in focal lineages. Statistical significance of a  
 454 likelihood ratio test compared to a model where only a single  $d_N/d_S$  ratio was used is indicated  
 455 by black ( $P < 0.05$ ) vs. gray ( $P > 0.05$ ) points. C) Natural log of the  $k$  parameter from the RELAX



456 analysis where  $\ln k < 0$  indicates relaxed selection in focal lineages and  $\ln k > 0$  indicates positive  
457 selection. Statistical significance is indicated as in B). Data are presented for individual  
458 mitochondrial genes and the concatenated set of all 13 mitochondrial protein-coding genes.  
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461

462 **Figure 3.** Selection on mitochondrial genes in lineages with energetic innovations. A) Topology

463 showing the number and distribution of focal lineages (in red; from left to right: snakes,

464 mormyroids, gymnotiforms, and primates) and reference lineages (in black). B)  $d_N/d_S$  ratios in

465 focal lineages compared to reference lineages, as in Figure 2. C) RELAX analysis where  $\ln k < 0$

466 indicates relaxed selection on focal lineages and  $\ln k > 0$  indicates positive selection. Statistical

467 significance and data presentation as in Figure 2.