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Body mass-corrected molecular rate for bird mitochondrial DNA

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24 Abstract

26 Mitochondrial DNA remains one of the most widely used molecular markers to reconstruct the
phylogeny and phylogeography of closely-related birds. It has been proposed that bird mitochondrial
genomes evolve at a constant rate of ~ 0.01 substitution per site per Million years, *i.e.* that they evolve
28 according to a strict molecular clock. This molecular clock is often used in studies of bird
mitochondrial phylogeny and molecular dating. However, rates of mitochondrial genome evolution
30 vary among bird species, and correlate with life-history traits such as body mass and generation time.
These correlations could cause systematic biases in molecular dating studies that assume a strict
32 molecular clock. In this study, we overcome this issue by estimating corrected molecular rates for birds.
Using complete or nearly complete mitochondrial genomes of 475 species, we show that there are
34 strong relationships between body mass and substitution rates across birds. We use this information to
build models that use bird species' body mass to estimate their substitution rates across a wide range of
36 common mitochondrial markers. We demonstrate the use of these corrected molecular rates on two
recently-published datasets. In one case, we obtained molecular dates that are twice as old as the
38 estimates obtained using the strict molecular clock. We hope that this method to estimate molecular
rates will increase the accuracy of future molecular dating studies in birds.

40

Introduction

42 Mitochondrial DNA is widely used to reconstruct the timescale of bird evolution using molecular
dating. The divergence times obtained from this approach have been used to examine the impact of life
44 history, geology, climate, and biogeography on the diversification of birds (e.g., Avise & Walker 1998;
Weir *et al.* 2009; Lanfear *et al.* 2010). However, the molecular dating methods used to derive the
46 timescale of bird evolution remain controversial.

Most molecular dating studies use a combination of fossil calibrations and relaxed molecular clocks to
48 infer divergence times from molecular data. Fossil calibrations provide information on the divergence
times of some nodes in the phylogeny, and relaxed molecular clocks allow divergence times of other
50 nodes to be estimated while allowing for variation in substitution rates among lineages. However, the

fossil record is scarce or completely lacking for most bird families and genera (Mayr 2009, 2013).
52 Consequently, molecular dating studies in birds often rely on strict molecular clocks, assuming a single
substitution rate that does not (or to a limited extent) vary over time and among lineages. This approach
54 allows molecular dates to be estimated entirely in the absence of fossil calibrations. The most
frequently employed method is the use of a divergence rate of 2% per million years (Myr). This rate
56 was proposed some time ago (Brown *et al.* 1979; Shields & Wilson 1987; Fleischer *et al.* 1998), and
recently reaffirmed by Weir & Schluter (2008) in a widely cited analysis in which they estimated a
58 divergence rate of 2.1% per million years (0.0105 subst/site/myr) for mitochondrial Cytochrome *b*
sequences in birds.

60 Although convenient, the use of strict molecular clocks in birds remains controversial (Lovette 2004;
García-Moreno 2004; Pereira & Baker 2006; Ho 2007; Nabholz *et al.* 2009; Nguyen & Ho 2016).
62 Widespread variation in rates of molecular evolution among species is not only predicted on empirical
grounds (e.g. Lehtonen & Lanfear 2014), but has also been repeatedly confirmed in empirical studies of
64 birds (Pereira & Baker 2006; Nabholz *et al.* 2009; Eo & DeWoody 2010; Pacheco *et al.* 2011; Jarvis *et al.*
2014; Nguyen & Ho 2016). Indeed, the broader picture is that variation in substitution rates is the
66 rule rather than the exception across the tree of life (Bromham *et al.* 1996; Welch *et al.* 2008; Nabholz
et al. 2008; Thomas *et al.* 2010; Lourenço *et al.* 2013; Qiu *et al.* 2014). Failing to account for this
68 variation may lead to systematically biased estimates of divergence times in molecular dating studies.
For example, if large birds tend to evolve more slowly than smaller birds, then the use of the 'standard'
70 strict molecular clock would lead to underestimates of divergence times for largest birds and
over-estimates of divergence times for smallest birds.

72 Across clades as diverse as vertebrates and angiosperms, substitution rates have repeatedly been shown
to correlate with life-history traits such as body mass, longevity, and generation time (Bromham 2009).
74 In birds, it has been shown that substitution rates in both the nuclear and mitochondrial genomes
correlate with life history traits (Nabholz *et al.* 2009; Jarvis *et al.* 2014) and species richness (Lanfear
76 *et al.* 2010; Eo & DeWoody 2010). These correlations suggest that the variation in substitution rates
among bird species is, to some extent at least, predictable. This presents opportunities to circumvent the
78 problem of using strict molecular clocks, because one might be able to use commonly measured
life-history traits, such as body size, to estimate variation in substitution rates among species. Using
80 this approach, one could define a 'corrected' molecular clock that accounts for some of the known
variation in substitution rates among lineages, even in the absence of fossil calibrations.

82

In this study, we used the complete or nearly mitochondrial genome of 475 birds species to produce a collection of corrected molecular rates for birds. We focus on body mass because it is known for most extant species of bird (Dunning 2007), and so provides the most convenient life-history trait from which to estimate corrected molecular rates for molecular dating studies. Conveniently, body mass could also be extrapolated using allometric relationship with skeletal measurements (e.g. Campbell and Marcus 1992; Field *et al.* 2013). We provide corrected molecular rates for third codon-positions, as well as for total substitution rates for each of the individual mitochondrial genes that are commonly used for molecular dating in birds. We hope that these corrected molecular rates will increase the accuracy of future molecular dating studies in birds.

92

Materials & Methods

94

Sequences, Alignments and Topology

We downloaded all of the complete or near-complete mitochondrial genomes available on NCBI/GenBank (<http://bcbl.nlm.nih.gov>) in May 2015, resulting in a dataset for 487 species. When multiple individuals were available per species, we selected the longest and/or the highest quality sequence (as inferred from the number of uncertainty codes). When all sequences were deemed to be of equal quality, we selected an individual randomly. We aligned the 12 H-stranded protein-coding genes (*i.e.* all the protein-coding genes except NADH-dehydrogenase 6, ND6) using BioEdit (Hall 1999), after excluding all nucleotides from the stop codons. We carefully checked all alignments by eye. We excluded 12 dubious sequences during alignment, resulting in alignments of 475 species (see Table S1). We estimated a Maximum Likelihood (ML) topology using RAxML (Model GTR+G; Stamatakis 2014). We used a constrained topology to follow Prum *et al.* (2015) (using option “-g” in RAxML) regarding the basal Neoavians relationships. We also repeated our analyses using a topology following Jarvis *et al.* (2014) that gave similar results. The topology and the alignments are available at DRYAD DOI.

110 **Life-history traits**

We obtained body mass data from Dunning (2007). When body mass was not available for a species,
112 we used the median body mass of the genus in Dunning (2007) except if another species of the same
genus was available in our dataset. Otherwise, we left that species' body mass unknown. For example,
114 body mass for the black-browed bushtit (*Aegithalos bonvaloti*) was not present in Dunning (2007) but
we left its body mass as unknown in our dataset because another *Aegithalos* (the long-tailed bushtit,
116 *Aegithalos caudatus*), for which a mass is available, is present in our dataset. Using this approach, we
obtained body mass estimates for 435 of the 475 species in our alignments.

118

Substitution rate estimation

120 In order to derive the most accurate molecular rate estimates across species, we used a two-step
approach inspired by Nabholz *et al.* (2008, 2009). This approach allows us to make the best use of the
122 relatively few and relatively old reliable fossil calibrations in birds (Benton *et al.* 2009; Ksepka &
Clarke 2015), while still estimating rates of evolution for fast-evolving mitochondrial DNA sequences
124 which typically would not be used together with such old fossil calibrations. First, we estimated
molecular divergence dates using amino-acid alignment and fossil-based calibrations (see “Molecular
126 dating”). Second, we used molecular divergence dates to calibrate the species-specific substitution rate
of nucleotide sequences. An outline of the method are presented in Supplementary Figure S1.

128 To limit the effect of saturation, we defined monophyletic groups of sequences for which the maximal
nucleotide divergence between any given pair of species was lower than 0.4 subst./site. To do this, we
130 used a custom R script built with the APE package (Popescu *et al.* 2012; R Core Team 2013), which led
to 84 independent monophyletic clades containing a total of 436 species. Inspection of a saturation plot
132 (a plot of phylogenetic divergences against the uncorrected pairwise genetic divergences) indicated that
saturation was moderate at 0.4 subst./site. A threshold set at 0.3 subst./site gave similar results.

134 Within each group, substitution rates were estimated using MCMCTREE (Yang & Rannala 2006;
Rannala & Yang 2007) implemented in PAML4 (Yang 2007). We applied an HKY model of
136 substitution with an autocorrelated relaxed clock model. We choose the HKY model because it is the
most complex model currently implemented in MCMCTREE. The analysis of each group was
138 calibrated using three estimates of the divergence date of the most recent common ancestor of each

group obtained from the molecular dating analysis based on amino-acid sequences (see below): the mean divergence date; the minimum 95% credibility interval of the divergence date; and the maximum 95% credibility interval of the divergence date. Using these three dates allows us assess the effects of the uncertainty of our divergence dates on our molecular rate estimates. The maximum 95% C.I.'s provide minimal age estimates and therefore maximal substitution rate estimates for each group, while the minimum 95% C.I.'s provide maximal age estimates and therefore minimal substitution rate estimates for each group. Because several groups contained only two sequences, we added an outgroup to all clades defined as the most closely related species in the phylogeny. For example, a clade contained the two *Halcyon* species (*Halcyon pileata*, *Halcyon sancta*) and we used the Pied Kingfisher (*Ceryle rudis*) sequence as outgroup. This outgroup was used to help in the estimation of substitution rates for ingroup sequences. Substitution rate estimates for outgroups were not used in downstream analyses, because they were occasionally quite divergent from ingroup sequences and so prone to issues associated with saturation.

Molecular dating

The molecular dating analyses using amino-acid sequences were performed with PHYLOBAYES version 4.1 (Lartillot *et al.* 2009) using the default site-heterogeneous mixture model (CAT) and a log-normal autocorrelated relaxed clock (option -ln) (Thorne *et al.* 1998). Autocorrelated relaxed clocks have been shown to outperform other relaxed-clock models (Lepage *et al.* 2007). The priors on divergence dates were uniform (Lepage *et al.* 2007). We ran two chains in parallel for at least 20000 steps for all the analyses, with a burn-in of 10000 steps. In all cases, the two chains gave similar results, suggesting that the MCMC analyses had converged. Convergences was also assessed visually and using “tracecomp” ensuring that the discrepancy statistic d between estimates parameters were below 0.4 for most parameters.

For the calibrations, we relied exclusively on fossils, in order to avoid potential problems linked to calibrations based on geographic distributions (Hedges 2005, 2011). We particularly avoided the controversial calibration based on the vicariance of New-Zealand to calibrate the first split within the passeriformes (Trewick & Gibb 2010; Mayr 2013; Gibb *et al.* 2015). Although less controversial, more recent biogeographic calibrations have been used to date the diversification of the Hawaiian honeycreepers (Lerner *et al.* 2011). We chose to not use these calibrations but instead use them as an

independent source of information to evaluate our analyses.

170 As the placement of some calibrations is ambiguous, we choose to test several combinations of fossil
calibrations summarized in Table 1, and discuss specific cases here. The Anseriformes fossil *Vegavis*
172 *iaai*, found in the Maastrichtian, would seem to provide a clear minimum bound for the
Neognathae/Paleognathae split (Benton *et al.* 2009; Ksepka & Clarke 2015). However, we chose to not
174 consider *Vegavis iaai* as an Anseriformes because its phylogenetic placement is ambiguous (Mayr
2009, 2013). For the maximum bound of the Neognathae/Paleognathae split, we followed the
176 proposition of Benton *et al.* (2009) and set it at 86.5 Myr. Alternatively, *Gansus yumenensis* from the
early Cretaceous of China (110 Myr) was used by Jetz *et al.* (2012) whereas Jarvis *et al.* (2014) used
178 the maximum boundary of the Upper Cretaceous (99.6 Myr).

The other minimum-bound calibrations we used in our molecular dating analysis all followed the
180 suggestions of Ksepka & Clarke (2015). Additionally, we constrained the Passeriformes/Psittaciformes
split between 53.5 and 65.5 Myr based on the first Eocene fossil of Australia (Boles 1997) and on the
182 stem Psittaciformes fossil *Pulchrupollia gracilis* (Prum *et al.* 2015). The maximum bound at 65.5 Myr
was used in all analyses except calibration set 1 (see Table 1) (Longrich *et al.* 2011; Prum *et al.* 2015) .
184 Finally, in one analysis (calibration set 4), we constrained the Oscines/Suboscines split between 28 and
34 Myr based on the first Suboscines from early Oligocene (28Myr, Mayr & Manegold 2006) and on
186 the Eocene/Oligocene limit (34Myr). This later maximum bound relies on the fact that all
Passeriformes fossils known from the Eocene are stem Passeriformes, therefore crown Oscines and
188 Suboscines may have appeared latter during the Oligocene (Mayr & Manegold 2004; Mayr 2013).

Table 1: Fossil calibration combinations used in molecular dating analyses

Calibration Sets	Taxon 1	Taxon 2	Maximum bound (Myr)	Minimum bound (Myr)
1, 2, 4	Neognathae	Paleognathae	86.5	66
3	Neognathae	Paleognathae	110	66
1, 2, 3, 4	Anseriformes	Galliformes	free	66
1, 2, 3, 4	Sphenisciformes	Procellariiformes	free	60.5
1, 2, 3, 4	Coraciidae	Alcedinidae	free	51.57
1, 2, 3, 4	Apodidae	Trochilidae	free	51
1	Psittaciformes	Passeriformes	free	53.5

2, 3, 4	Psittaciformes	Passeriformes	65.5	53.5
4	Oscines	Suboscines	34	28

In order to compare the results obtained using the four different calibration sets we propose (Table 1), we selected five divergence dates within the phylogeny (Table 2) for which we have independent evidence with which to assess the accuracy of our own analyses: (i) the first divergence within the extant Hawaiian honeycreeper which was inferred to occur 6 Myr (Lerner *et al.* 2011); (ii) the Sittidae (represented by *Sitta*) / Troglodytidae (represented by *Henicorhina* and *Campylorhynchus*) split for which the maximum bound is provided by the early Miocene (20.5–18 Myr) fossil of a Certhioidea (a clade containing the Sittidae and Certhidae) (Manegold 2007); (iii) the Oscines/Suboscines split for which a maximum bound is provided by the first Suboscines fossils (~28 Myr) (Mayr & Manegold 2006); (iv) the Stercorariidae / Alcidae split; and (v) the Jacanidae / other Scolopaci split; for both (iv) and (v) Smith (2015) provides minimum calibrations points that could be compared with our analyses (Table 2).

Corrected rate model

All the statistical analyses were performed with R (R Core Team 2013). In the linear regression, body mass and substitution rate were log transformed (logarithm of base 10) and parameters of the linear model were estimated using the R formula $\text{lm}(\log_{10}(\text{Rate}) \sim \log_{10}(\text{Mass}))$. We repeated this analysis accounting for the relatedness among species using phylogenetic generalised least squares (PGLS) (Grafen 1989; Garland *et al.* 2000), and phylogenetic independent contrast (PIC) (Felsenstein 1974), implemented in APE (Paradis 2011). However, the parameters estimated using these methods could not be used to obtain corrected substitution rates using an independent species' body mass.

Substitution rate variation among protein-coding genes

In order to test for variation in substitution rates among different protein-coding genes, we performed additional molecular dating analyses for all genes individually, except for the very short ATP8 and ND4L genes. This led to an additional 20 molecular dating analyses: all codon positions and third codon positions for each of 10 protein-coding genes individually. For each of these analyses, we

estimated the branch lengths using the F84 model implemented in BASEML (Yang 2007). We did not use the HKY model here because we could not optimize it using BASEML on some datasets. However, the F84 model is conceptually similar to the HKY model used in MCMCTREE analyses. To avoid overparametrisation due to the short alignments, we fixed the value of the Kappa parameter (the transition to transversion ratio) to the value estimated using the whole dataset (Kappa = 8). We also used the gamma distribution of rate variation among sites only in the analyses using all codon positions. We obtained substitution rates by dividing the terminal branch lengths by the appropriate divergence times estimated in the molecular dating analyses. In this case, very small branches can be associated with extremely high variance, we therefore excluded branch lengths shorter than 0.01 substitution per site. The same sets of species were analyzed for every gene meaning that if a species has a branch shorter than 0.01 for on particular gene it was excluded for all, leading a set of N = 292 species. We did not use MCMCTREE for these analyses because many of these individual-gene datasets were small and the MCMC chain failed to converge.

To statistically compare substitution rates among protein-coding genes, we used an ANOVA with single factor repeated measures design with a model reflecting that the genes are nested within the 84 independent monophyletic clades (R formula : $\text{lm}(\text{Rate} \sim \text{Gene} + \text{Gene:Clade})$). We also tested a model where Clade was a random effect (R formula : $\text{lme}(\text{Rate} \sim \text{Gene}, \text{random} = \sim 1 | \text{Clade/Gene})$). Finally, we tested the rate differences between each pair of genes using pairwise t-tests with adjusted p-values using a Bonferroni correction.

Working examples

We applied our method to two recently published datasets including relatively large and small birds. The first datasets was composed of 16 ND1 sequences of *Circus* Harriers (body mass ~ 500g; Oatley *et al.* 2015). For this analysis, we used the phylogenetic tree estimated from these sequences in the original study (Figure 2 of Oatley *et al.* 2015).

The second dataset was composed of CYTB and ND2 sequences of the Buff-throated Woodcreeper complex (genus *Xiphorhynchus*, body mass ~ 40g; Rocha *et al.* 2015). We restricted the analysis to one sequence per taxon (as presented in Appendix A and Appendix B of Rocha *et al.* 2015) leading to a total of 11 sequences. As above, we used the phylogenetic tree estimated from these sequences in the

246 original study (Figure 1 of Rocha *et al.* 2015).

In both examples, sequences were aligned using muscle (Edgar 2004) and checked by eye. Sites
248 containing missing data were excluded. Branch lengths were estimated exclusively from the third
codon position. We applied a molecular clock model in BASEML (model F84, Kappa parameter fixed
250 to 8.0, parameter “clock = 1”). Then we scaled the branch lengths of the tree using the body mass
corrected substitution rate for each clade (i.e. a single scalar is used for the entire tree). Finally, we
252 estimated branch lengths from all codon positions for both datasets in order to compare our estimates
with the commonly-used 2% per Myrs strict molecular clock rate. Alignments, phylogenies and the R
254 script to convert branch lengths to divergence dates are provided at DRYAD.

256 **Results**

Molecular dating

258 Most fossil-based calibrations available are relatively deep in the bird phylogeny (Benton *et al.* 2009;
Jarvis *et al.* 2014; Ksepka & Clarke 2015; Prum *et al.* 2015), and are not suited to the fast evolving
260 nucleotide sequences of the mitochondrial genome (Nguyen & Ho 2016). To make use of these ancient
calibrations, we performed a molecular dating analysis with the amino-acid sequences because they are
262 less prone to saturation (Brown *et al.* 1982). We used four sets of calibrations (Table 1) reflecting the
current uncertainty surrounding fossil calibrations for molecular dating analyses of birds. We also
264 tested two topologies reflecting alternative hypotheses regarding the basal Neoavian divergences
(Jarvis *et al.* 2014; Prum *et al.* 2015). We obtain very similar dates between topologies (results not
266 shown). Hereafter, we present only the results obtained using the Prum *et al.* (2015) topology.

Globally, the calibration sets 1, 2 and 3 provide very similar divergence dates that are all compatible
268 with independent fossil data (Table 2). The Oscines/Suboscines divergences are, however, 30 to 35 Myr
older than the first known fossils (Mayr & Manegold 2004; Mayr 2013). Our analyses also provide
270 divergence dates that are more than twice as old as the dates of Lerner *et al.* (2011) for the
diversification of Hawaiian honeycreepers (Table 2).

272 **Table 2: Comparison of molecular dating analyses with independent fossils or independent
analysis. Mean dates are indicated and 95% C.I. are provided within brackets.**

# Calibration sets	Hawaiian honeycreepers	Oscines / Suboscines	Sittidae / Troglodytidae	Stercorariidae / Alcidae	Jacanidae / other Scolopaci
Minimum fossil bound (Myr)	6	28	18	34.2	30
1	15.8 (12.1, 19.9)	63.9 (57.8, 69.9)	35.2 (29.9, 40.6)	47.9 (39.6, 56.5)	41.8 (32.2, 51.4)
2	13.4 (10.1, 16.9)	57.1 (54.9, 59.1)	30.3 (26.7, 33.6)	43.9 (35.8, 49.4)	37.6 (28.7, 46.0)
3	14.3 (10.9, 18.0)	57.4 (55.1, 59.3)	31.6 (27.3, 35.3)	42.7 (36.4, 48.9)	36.9 (28.1, 45.5)
4**	7.2 (5.8, 8.8)	33.9 (33.5, 34.0)**	19.0 (17.3*, 21.0)	41.2 (34.9, 46.5)	35.1 (26.6*, 42.3)

*Indicates values that are in conflict with the fossil records.

**This analysis, the Oscines / Suboscines split was constrained to occur between 28 and 34 Myr

Calibration set 4 provides younger dates than the other calibration sets, particularly within the Passeriformes. This is expected as this analysis includes an additional calibration bound that constrained Oscines / Suboscines split to occur between 28 and 34 Myr. This is about half the age of this split recovered with other calibration sets that do not calibrate this node (Table 2). This analysis provided dates that are in agreement with those of Lerner *et al.* (2011) but the minimum 95% credibility intervals (C.I.) are younger than the fossils records for the Sittidae / Troglodytidae split and for the Jacanidae / other Scolopaci split although the discrepancy is modest (Table 2, 17.3 vs 18 Myr and 26.6 vs 30 Myr respectively).

It is difficult to favor one analysis over the others, therefore, we choose to consider both calibration set 2 (which is representative of the set 1, 2 and 3) and calibration set 4 for the following analyses.

A mass-corrected molecular rate

We selected 84 independent monophyletic groups containing a total 437 species (Table S2). These groups match a criterion of limited divergence in order to accurately estimate substitution rates. Substitution rates were calibrated for each group using divergence dates obtained from the molecular dating using the fossil calibration sets 2 and 4 (see method). Hereafter, we will refer to them as

“analysis 2” and “analysis 4” respectively.

294 For analysis 2, the median third codon substitution rate was 0.0142 subst/sites/Myr (min = 0.0035,
max = 0.0306). For analysis 4, the median third-codon substitution rate was 0.0165 subst/site/Myr (min
296 = 0.0042, max = 0.0566). In both cases, the substitution rate varies by more than one order of magnitude
among species.

298 To compare our approach with the widely-used divergence rate of 0.01 substitutions per site per million
years, which was originally estimated from all codon positions, we also inferred substitution rates using
300 all codon positions. For analysis 2, the median substitution rate was 0.0035 subst/site/Myr (min =
0.0010, max = 0.0090) with all species having a rate below 0.01. For analysis 4, the median
302 substitution rate was 0.0042 subst/site/Myr but the distribution obtained overlapped extensively with
the 0.01 (min = 0.0011, max = 0.0158). The passerines, for example, have a median substitution rate
304 that is very close to it (median = 0.009 subst/site/Myr).

Both third codon and all position substitution rates are strongly negatively correlated with body mass
306 (Table 3). The R^2 values are higher when substitution rates are estimated from third-codon positions
(0.46 and 0.60 for the analysis 2 and 4 respectively, Figure 1) than when they are estimated from all
308 codon positions (0.35 and 0.51 for the analysis 2 and 4 respectively). We speculate that this could result
from the effects of natural selection on substitution rates. These effects are likely to be stronger on
310 codon positions 1 and 2 than on third codon positions, and may weaken the relationship between body
mass and substitution rates. However, regardless of the cause of the higher R^2 for third codon positions,
312 it implies that body mass is a better predictor of third codon position substitution rates than of
substitution rates derived from all codon positions. As such, body mass corrected rates will be more
314 accurate if they are derived using substitution rates estimated from third codon positions only, and so
we focus on deriving corrected rates for third codon positions only.

316 Both the GLS and PIC analyses, which control for phylogenetic non-independence among species,
confirmed a significant negative correlation between substitution rate and body mass (Table S3),
318 demonstrating that the relationship between body mass and substitution rate is robust to control of the
phylogenetic relationship among species. A similar conclusion was made by Nabholz *et al.* (2009).

320 The slope and the intercept of the linear models comparing third codon position substitution rates and
body mass are presented in Table 3. These models allow us to predict a species' third codon
322 mitochondrial substitution rate given its body mass using the following equation:

$$\text{Substitution rate} = \frac{10^{(\text{slope} * \log_{10}(\text{Mass}) + \text{intercept})}}{100}$$

For example, using the parameters of analysis 2, a bird of 10g (e.g. small passerines – some White Eyes: Zosteropidae, Sunbirds: Nectariniidae) is predicted to evolve at $(10^{(-0.145 * \log_{10}(10) + 0.459)})/100$ 0.0206 subst/site/Myr for third codon position. In contrast, a birds of 1000 g such as the Mallard (*Anas platyrhynchos*), the Kelp gull (*Larus dominicanus*) or a medium size raptors such as the Crested Serpent Eagle (*Spilornis cheela*) for example, will evolve 2 times slower at 0.0106 subst/site/Myr for third codon position. These values convert to divergence rates of 4.2% and 2.1% per Myr respectively. Analysis 4 gave linear models with steeper slopes than analysis 2, which creates a larger difference between the predicted substitution rates for any given pair of species. For example, in Analysis 4, birds of 10g and 1kg are predicted to have substitution rates of 0.0368 and 0.0118 subst/site/Myr respectively (7.4% and 2.4% divergence per Myr).

Finally, to take the uncertainty of molecular dating into account, we repeated the substitution rate estimation using secondary calibration points taken from the minimum and the maximum 95% C.I. of the estimated divergences dates. The maximum 95% C.I. provide minimal divergence and, therefore, an estimated maximum for the substitution rate whereas the minimum 95% C.I. dates provide a minimal substitution rate (Table 3).

Table 3: Parameter of the linear model: $\log_{10}(\text{Rate}) \sim \log_{10}(\text{body mass})$. Substitution rates were divided by 100 in the model. Maximum and minimum 95% C.I. correspond of estimates made using 95% C.I. of molecular dating.

Positions	Calibration sets	R ²	Mean slope; intercept	Maximum 95% CI slope; intercept	Minimum 95% CI slope; intercept
3 rd	2	0.46	-0.145; 0.459	-0.143; 0.559	-0.141; 0.367
3 rd	4	0.60	-0.247; 0.813	-0.243; 0.905	-0.246; 0.730
All	2	0.35	-0.122; -0.185	-0.123; 0.076	-0.118; -0.274
All	4	0.51	-0.227; 0.177	-0.220; 0.266	-0.224; 0.089

Substitution rates differ among Protein-coding genes

For each of the protein-coding genes in our dataset (except ND6, ND4L and ATP8), we estimated

346 substitution rates from both third codon positions alone and all codon positions combined. The results
from analyses 2 and 4 are qualitatively similar, so we only present the results from analysis 2 here.
348 Using all codon positions, we detected significant variation in substitution rates among genes (linear
model, Gene effect : $p < 0.001$). COX1, COX2, COX3 and CYTB have a lower substitution rate than the
350 ND genes and ATP6 (Figure 2a, pairwise t-test). The fastest-evolving gene (ND2, median rate =
0.0081) evolves, on average, 1.5 faster than the slowest (COX2, median rate = 0.0055) (see Table S3
352 for details).

Using third codon positions, we also detected significant variation in substitution rates among genes
354 (linear model, Gene effect : $p < 0.001$). As for all codon positions, COX proteins are generally slower
than ND and ATP6 (Figure 2b). The range of variation is lower, with the fastest gene (ND1, median
356 rate = 0.0150) evolving 1.2 faster than the slowest (COX3, median rate = 0.0121).

To take these variations into account, we computed the difference between the median rate of each gene
358 individually with the full alignment (Table S4). These values could serve as correction factor to the
mass-corrected substitutions when using a reduced sets of genes.

360

Working examples

362 *Circus* Harriers

We obtained the body mass of 14 out of 16 species (*C. hudsonius* and *C. spilothorax* are missing) of
364 harriers in the original dataset. body mass appears homogeneous in this clade (mean = 507g, sd = 132g)
which is ideal to apply a single mass-corrected rate for the whole phylogeny. Moreover, the molecular
366 divergence is moderate, with the maximal divergence between any two species at 14%. It is important
to limit the divergence as third codon position can saturate rapidly due to their fast rate of evolution,
368 leading to an inaccurate branch length estimates (Nguyen & Ho 2016). Using the parameters from
Table 3, the mass-corrected substitution rates vary between 0.0116 (0.0097 – 0.0149) subst/sites/Myr
370 for third codon position and 0.0140 (0.0116 – 0.0177) subst/sites/Myr for third codon position for the
calibration sets 2 and 4 respectively. This rate leads to divergence dates that are approximately twice as
372 old as the dates that would be obtained by assuming the widely-used molecular clock rate of 2.1%
divergence per Myr (Table 4). The first split within the genus (Node B) is estimated to have occurred at
374 10.3 to 15.8 Myr following by the diversification of “steppe” Harrier (Node C, D, and I, Oatley *et al.*

2015). Applying the gene specific rate, the discrepancy would have been even greater because ND1 evolves 5-7.5% faster than the rate estimated with all the protein-coding genes (Table S3). Although much older, these dates are still largely in agreement with the appearance of open habitat and C4 grasslands ecosystems during mid-Miocene (Edwards *et al.* 2010). Our estimates also support a recent origin of the Marsh harrier clades (Node E) of between 2.9 and 4.4 Myr.

Table 4: Divergence times and confidence intervals according to body mass corrected substitution rate (Calibration sets 2 and 4) and to the 2.1% of divergence per Myr. The node are presented in the figure 2 of Oatley *et al.* (2015)

Node	2.1 % ($\pm 0.1\%$)	Calibration set 2	Calibration set 4
B	6.3 (6 - 6.6)	13.1 (10.3 - 15.8)	11 (8.7 - 13.2)
C	4.7 (4.5 - 4.9)	9.7 (7.6 - 11.6)	8.1 (6.4 - 9.7)
D	4.2 (4 - 4.4)	8.3 (6.5 - 10.1)	7 (5.5 - 8.4)
E	2 (1.9 - 2.1)	3.7 (2.9 - 4.4)	3.1 (2.4 - 3.7)
F	1.0 (0.9 - 1)	1.7 (1.3 - 2.0)	1.4 (1.1 - 1.7)
G	0.5 (0.5 - 0.6)	0.8 (0.6 - 0.9)	0.6 (0.5 - 0.8)
H	0.1 (0.1 - 0.1)	0.1 (0.1 - 0.2)	0.1 (0.1 - 0.1)
I	5.5 (5.2 - 5.7)	12 (9.4 - 14.5)	10.1 (7.9 - 12.1)
J	1.7 (1.7 - 1.8)	3.5 (2.7 - 4.2)	2.9 (2.3 - 3.5)
K	1.3 (1.2 - 1.4)	2.5 (1.9 - 3)	2.1 (1.6 - 2.5)
L	0.9 (0.9 - 1)	2.0 (1.5 - 2.4)	1.6 (1.3 - 2)

Buff-throated Woodcreeper complex *Xiphorhynchus*

We obtained the body mass of 6 out of 7 species of *Xiphorhynchus* (*X. eytoni* is not available) in the original dataset. The maximal molecular divergence between any two species is 12%. As for the Harrier, the body mass appears very homogeneous in this clade (mean = 48.6g, sd = 8.8g). Using the parameters from Table 3, the mass-corrected substitution rates vary between 0.0163 (0.0135 – 0.0208) subst/sites/Myr for third codon position and 0.0249 (0.0207 – 0.0313) subst/sites/Myr for third codon

position using for calibration sets 2 and 4 respectively. The mass-corrected rate leads to divergence dates that are close to those obtained using the widely-used divergence rate of 2.1% per Myr (Table 5) and, therefore, are largely congruent with the dates proposed in the original publication that include paleontological and fossil calibrations as well as the molecular clock calibration (Rocha *et al.* 2015).

Table 5: Divergence times and confidence intervals according to body mass corrected substitution rate (Calibration sets 2 and 4) and to the 2.1% of divergence per Myr. The node are presented in the figure 3 of Rocha *et al.* (2015)

Node	2.1% ($\pm 0.1\%$)	Calibration set 2	Calibration set 4
I	3.9 (3.8 - 4.1)	6.0 (4.7 - 7.3)	3.9 (3.1 - 4.7)
II	3.4 (3.2 - 3.6)	5.4 (4.2 - 6.5)	3.5 (2.8 - 4.2)
III	2.2 (2.1 - 2.3)	3.1 (2.5 - 3.8)	2.1 (1.7 - 2.5)
IV	1.3 (1.2 - 1.3)	2.1 (1.7 - 2.6)	1.4 (1.1 - 1.7)
V	0.4 (0.4 - 0.5)	0.6 (0.5 - 0.7)	0.4 (0.3 - 0.5)

Discussion

In the present study, we propose a simple model to predict mitochondrial substitution rates from species' body mass. In line with several previous studies (Pereira & Baker 2006; Brown *et al.* 2008; Nabholz *et al.* 2009; Lanfear *et al.* 2010; Eo & DeWoody 2010; Pacheco *et al.* 2011), we detected extensive and statistically significant variation in substitution rates among bird species. Using body mass alone, we were able to predict up to 60% of the variation in observed substitution rates, suggesting that using body mass corrected molecular rates is superior to a strict molecular clock approach.

The extensive substitution rate variation we describe is smaller than the one reported by Nabholz *et al.* (2009). Nabholz *et al.* (2009) reported a median of 0.018 subst/sites/Myr with a variation from 0.003 and 0.09 subst/sites/Myr. This discrepancy could simply be explained by the fact that Nabholz *et al.* (2009) used only CYTB for molecular dating and substitution rate estimation in contrast to all the H-strand encoded protein-coding genes therefore leading to higher sampling variance in the substitution process in the earlier study.

412 Our estimates of mitochondrial substitution rates are often lower than the commonly-used divergence
rate of 2% per million years, although, they are largely concordant with the estimates of Pereira and
414 Baker (2006) (range from 0.0009 to 0.012 subst/sites/Myr) and Pacheco et al. (2011) (average rate per
genes range from 0.0027 to 0.0068 subst/sites/Myr). It was only in the analysis constraining the
416 divergence between the Oscines and Suboscines to be younger than 34 Myrs (Analysis 4) that a
substantial number of species (mostly passerines) have a divergence rates close to or above 2% per
418 million years. The discrepancy between the analyses reflect the current uncertainty surrounding fossils
calibrations used to estimate the timescale of avian diversification (Mayr 2013; Cracraft *et al.* 2015;
420 Mitchell *et al.* 2015). For example, analyses with calibration sets 1, 2 and 3 provide an age for the
Oscines / Suboscines divergence that is 30 to 35 Myr years older than the fossil record. This
422 discrepancy may seem surprising because no stem Passeriformes are known from the Paleocene, let
alone the derived Oscines and Suboscine representatives (Mayr 2009). However, the fossil record of
424 passerines could be biased towards missing the earliest fossils, because most known deposits exist in
the northern hemisphere whereas both crown and stem Passeriformes likely originated in the southern
426 hemisphere (Boles 1997; Ericson *et al.* 2002). Our analyses 1, 2, and 3 suggest that the diversification
of Hawaiian Honeycreepers predate the oldest Hawaiian island (Kauai-Miihau). Thus, our analyses
428 support either that the diversification started before the emergence of the oldest island or, that at least
two independent colonization events have occurred. Both scenarios are possible as older islands existed
430 before Kauai-Miihau (Price & Clague 2002) and many examples of old taxa on young islands are
known (Heads 2011), although the latter could also result from the extinction of the mainland sister
432 taxa.

Our analyses show that the correlations between body mass and substitution rate are stronger when
434 substitution rates are estimated exclusively from third codon positions. This could be because third
codon positions are less affected by natural selection than first and second codon positions. Selective
436 constraint acting on mitochondrial genes is known to be associated with speed of movement (Shen *et al.* 2009) and island/mainland status (Johnson & Seger 2001; Woolfit & Bromham 2005), a variable
438 related to the effective population size. Positive selection has also been detected in vertebrate
mitochondrial evolution (Grossman *et al.* 2004; Castoe *et al.* 2008, 2009) although not, to date, in
440 birds. Positive selection could episodically inflate the substitution rate. Regardless of the reason for the
difference in the strength of association between body mass and substitution rate estimates, we suggest
442 using molecular rates estimated from third codon positions when using body mass corrected molecular

rates, since it is these rates which can be most accurately predicted from body mass data. Our analyses
444 reveal significant variation in substitution rates among mitochondrial protein-coding genes. Using
substitution rates estimated from all codon positions, we show that NADH-dehydrogenase (ND,
446 proteins of the complex I) genes evolved faster than Cytochrome Oxidase (COX, proteins of the
complex IV) and CYTB genes. These results are in line with those of Eo & DeWoody (2010), Smith &
448 Klicka (2010), Pacheco *et al.* (2011), Lerner *et al.* (2011) and Nguyen & Ho (2016). Eo & DeWoody
(2010) and Pacheco *et al.* (2011) report a variation among genes that is higher than in our analysis (e.g.
450 ND2 being 2.2 times faster than COX1 in Pacheco *et al.* 2011). Using datasets composed of passerines,
Nguyen & Ho (2016) also detected fast evolution for ND genes, however, CYTB was reported as
452 clearly the slowest genes which is not the case in our analysis. The observed variation might be a
consequence of the variation in evolutionary constraint among genes, since the ND genes are known to
454 be less constrained than the COX genes (Mishmar *et al.* 2003; Nabholz *et al.* 2013). Interestingly, we
also detected extensive variation of third codon position substitution rates among protein-coding genes.
456 The variation among genes could be explained in two ways: i) a difference in mutation rate among
genes and ii) selection acting on synonymous sites. Neither GC content nor the positions of the protein
458 in the genome (that is linked to the time spend single stranded during, Reyes *et al.* 1998) explain the
observed difference in substitution rate among genes. Selection on synonymous sites is a possibility, for
460 example, through a selection on codon usage driven by the large variation of expression level among
protein-coding genes (Nabholz *et al.* 2013). Additionally, indirect evidence of selection on synonymous
462 sites is also provided by mutations linked to Human disease. Among the 271 point mutations located
within the protein-coding genes that are known to cause diseases in humans, nine are synonymous
464 mutations (Lott *et al.* 2013).

Finally, we re-analysed two datasets to illustrate the principle of our method. These analyses show that
466 in some cases, particularly with non-passerine birds, the assumption of the widely-used divergence rate
of 2% per million years can give very different answers from using the body mass corrected molecular
468 rates we derive here. We emphasize three additional points as a guideline to users of our corrected
molecular rates. First, the species analysed should have a limited molecular divergence. An arbitrary
470 threshold could be a pairwise molecular divergence below 1.0 subst/site for the third-codon position,
which corresponds to a divergence of 0.3-0.4 subst/site (that is 30 to 40% divergence) for all codon
472 position. When divergence is higher, we recommend splitting the dataset into smaller clades and
analysing each clade independently. Second, when few loci are available, over-parametrisation of the

474 model of molecular evolution could be an issue. Particularly, we noted that the Kappa parameter in our
empirical analyses increased to unrealistic values (>100) in some small datasets, leading to
476 unrealistically inflated branch length estimates. We therefore recommend fixing this parameter to a
reasonable value *a priori* – for example, the one estimated using all codon positions – when only a few
478 loci are available for analysis. Third, the within-clade body mass should be rather homogeneous among
the species of a clade (this is likely when analysing clades of limited molecular divergence). This will
480 help to ensure that applying a single correction factor the molecular branch-lengths is warranted. If
body mass varies widely within a clade, the substitution rate is unlikely to be accurately approximated
482 using a single molecular clock estimated from the average body mass of that clade. In this case, we also
recommend splitting the dataset into monophyletic clades of species with relatively homogeneous body
484 mass, and analysing each clade independently.

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492 **Data accessibility**

- Alignments, phylogeny, and body mass data for the main dataset, the two working examples and R
494 script to compute the molecular date using species body mass : Dryad doi:10.5061/dryad.1040d

496 **Legends**

Figure 1: Correlation between body mass and third-codon position substitution rates. The dotted green
498 line indicates an approximation of the rate corresponding to a 2% divergence per million years.

Figure 2: Pairwise comparison of substitution rates between protein-coding genes. Colour indicates the
500 direction and the intensity of the difference. A deep blue color indicates that the gene on the X-axis
evolves at a faster rate than the gene on the Y-axis whereas a white color indicates the opposite. A star

502 indicates when the difference is statistically significant (Pairwise t-test with p value < 0.01 after Bonferroni correction).

504 **Figure S1:** Schematic descriptions of two-steps method. A) The molecular dating is performed on the whole phylogeny using amino-acids sequences and fossil-based calibrations; B) The species-specific
506 substitution rate is estimated in groups of moderate divergence (here group #40) using third-codon positions. The blue dot represents the calibration obtained from analysis A and used to estimate
508 divergence date in analysis B.

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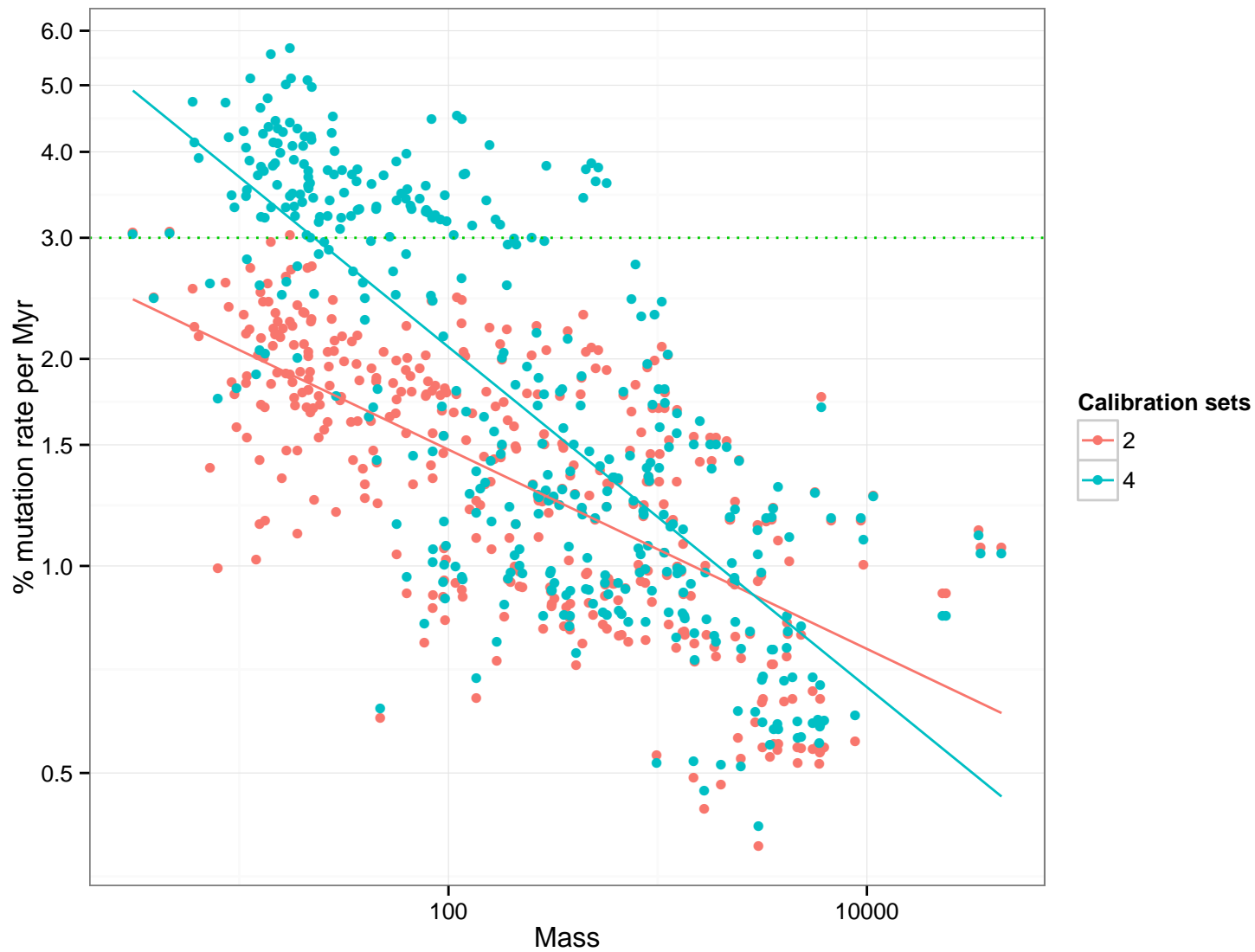
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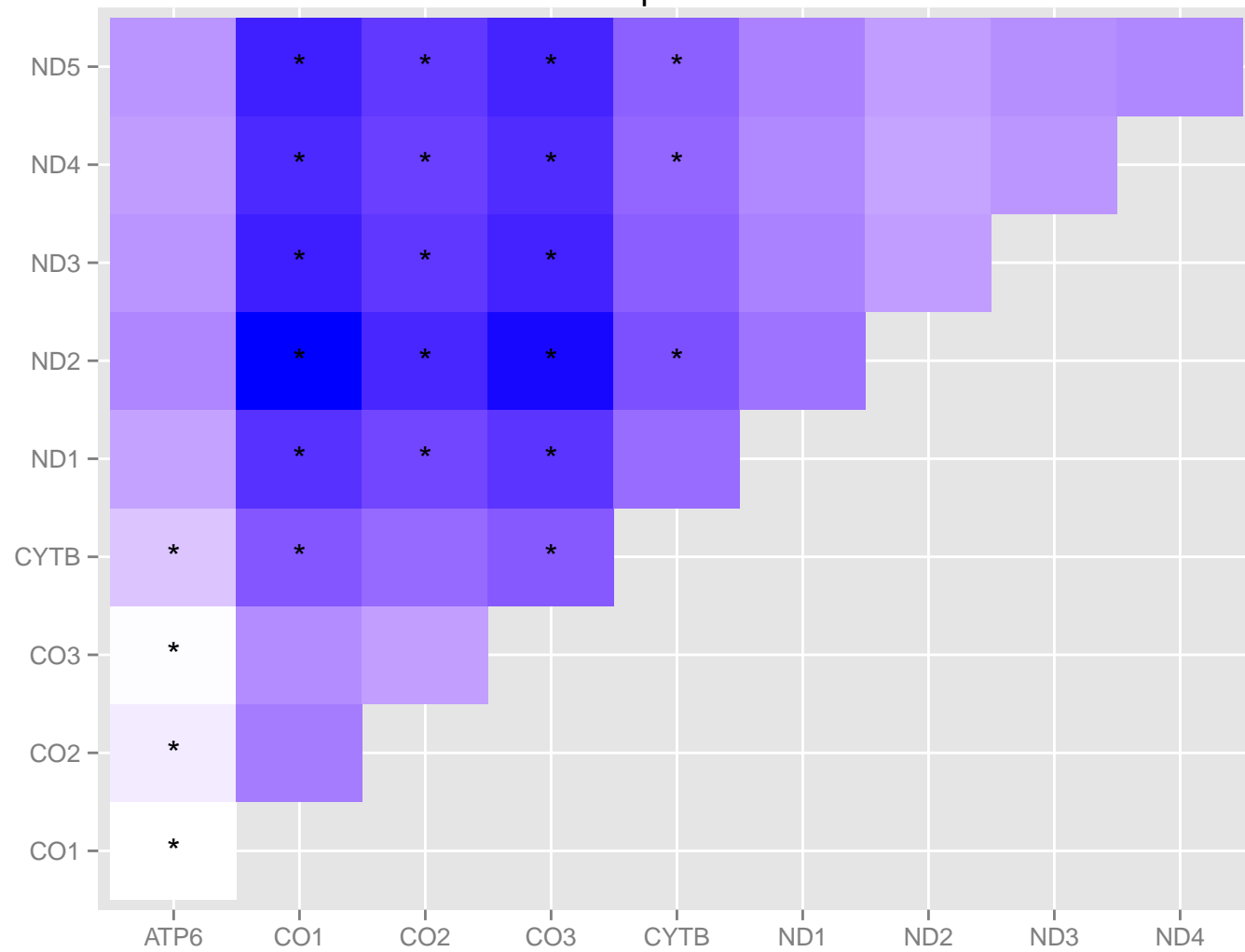
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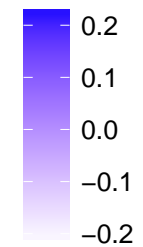
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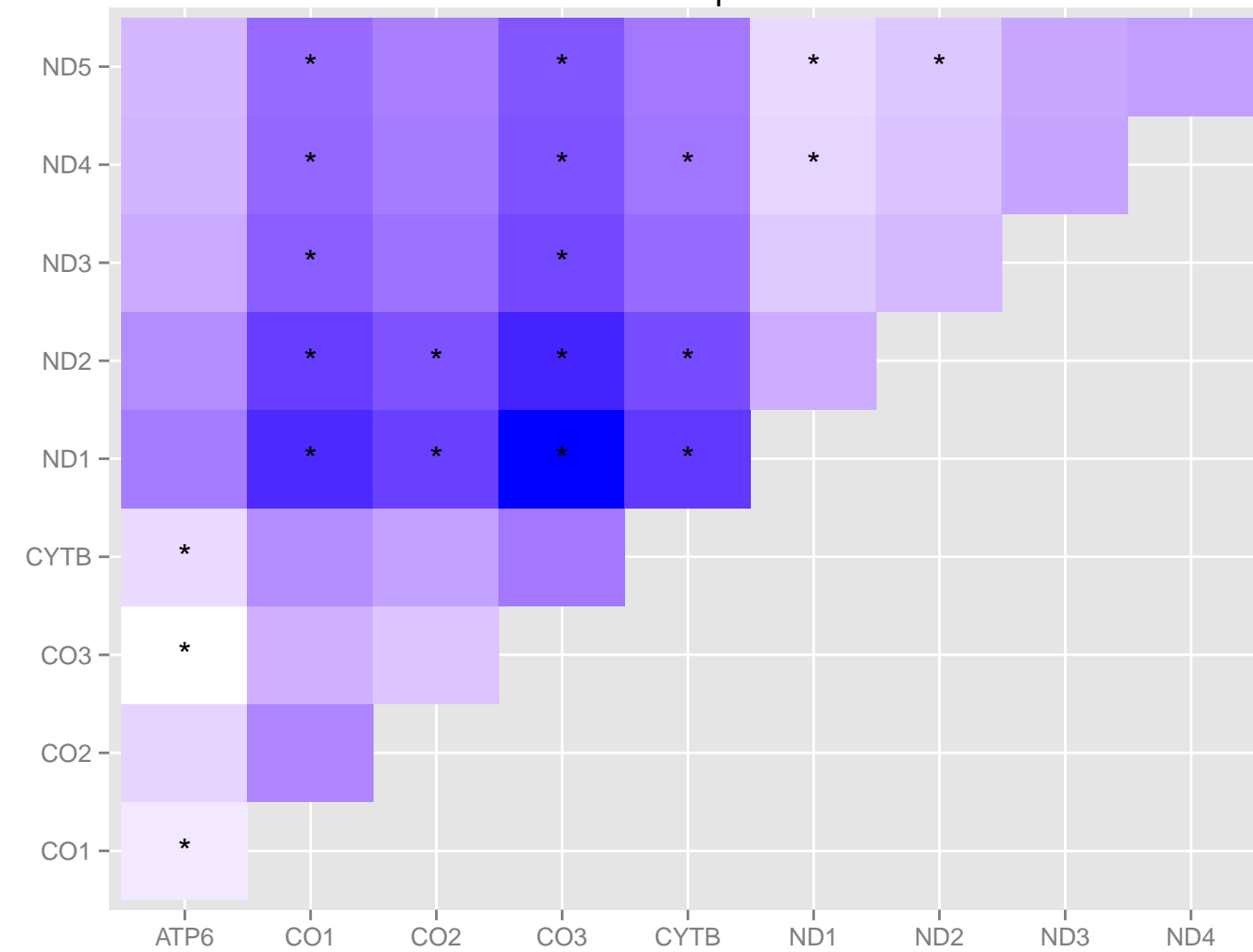
All position



Rate difference (Gene Y - Gene X)



3rd-codon position



Rate difference (Gene Y - Gene X)

