1 **Hyper-specialized bamboo lemurs possess a reduced suite of xenobiotic-metabolizing**

2 **cytochrome P450 genes.**

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10 **Introduction**

11 Among mammals, a large proportion of xenobiotic compounds are transformed or
12 detoxified by enzymes within the cytochrome P450 superfamily. Because of this role that P450s
13 play in mediating potential environmental toxins and because species vary in their diets and
14 exposure to such compounds, selective pressure has induced non-random additive and
15 adaptive changes in genetic sequence and copy number among species (Chaney et al., 2018,
16 2020; Ingelman-Sundberg, 2022; Kawashima & Satta, 2014; Kirischian et al., 2011; Skopec et al.,
17 2022). The effect of evolving toxin resistance has been especially well-documented for pest
18 insect species because of the economic importance of pesticides and insects' evolutionary
19 responses to those substances (Wondji et al. 2009; Feyereisen 2011; Bass et al. 2014; Wang et
20 al. 2014; Walsh et al. 2018; Joussen and Heckel 2021). Studies of P450 gene adaptation outside
21 of pest insects have often focused on dietary xenobiotics or plant secondary metabolites
22 (PSMs). For example, research on a model system comprising several species of woodrat
23 (*Neotoma* spp.) has shown that PSMs common in their diet (*e.g.*, alkaloids, terpenoids) are
24 metabolized especially by CYP2B enzymes and, to a minor extent, other xenobiotic-
25 metabolizing enzymes (Haley et al., 2007a, 2007b; Orr et al., 2020; Wilderman et al., 2014).

26 Comparisons between specialist and generalist species of these woodrats have shed
27 light on the effects that diet can have on P450 evolution. For instance, the generalist woodrat
28 species (*N. albigula*) was found to have lower copy number but greater sequence diversity of
29 *CYP2B* relative to a specialist (*N. stephensi*). This greater diversity of the generalist is consistent
30 with the broader array of PSMs with which the generalist must contend (Kitanovic et al., 2018).
31 The increased copy number of the specialist, in contrast, is in line with the prediction that genes

32 important for coping with the specialist's narrow diet will be subject to *additive* evolutionary
33 processes (*e.g.*, gene duplication, positive-selection) that will enhance the species' ability to
34 mitigate a narrow range of metabolically harmful compounds (Dearing et al., 2005; Freeland &
35 Janzen, 1974). This is taken to an extreme for specialized koala (*Phascolartcos cinereus*), in
36 which the key xenobiotic metabolizing *CYP2C* subfamily has reached a copy number of 31 via
37 serial duplication (Johnson et al., 2018).

38 Mammalian dietary specialization has also been linked to patterns of gene loss (Blumer
39 et al., 2022; Hecker et al., 2019; Itoigawa et al., 2019; Lauterbur, 2019; Marciniak et al., 2021;
40 Wagner et al., 2022). For example, vampire bats (genera *Desmodus, Diaemus,* and *Diphylla*)
41 have lost sweet and umami taste receptors (Zhao et al., 2010b; Zhao et al., 2012) and have
42 experienced reduction of their bitter-taste sense through pseudogenization of *TAS2R* genes
43 (Hong & Zhao, 2014). Interestingly, losses of gustatory genes have also been found for
44 cetaceans, which have lost nearly all taste-receptor genes apart from those for salty taste (Feng
45 et al., 2014), and for pandas (*Ailuropoda melanoleuca*), which have lost their umami taste-
46 receptors (Zhao et al., 2010a). Theory predicts that genes unimportant to a specialist's dietary
47 adaptation will be subject to a suite of *subtractive* evolutionary processes (*e.g.,* accumulation of
48 deleterious mutations, pseudogenization, or loss of protein coding region or entire gene). Gene
49 loss could either be for adaptive reasons (see Olson, 1999) or because of a release of selective
50 pressure (see Blumer et al., 2022; Huelsmann et al., 2019). Despite these examples and the
51 theory surrounding them, subtractive mechanisms such as gene loss have remained relatively
52 understudied compared to additive mechanisms such as gene duplication and positive or
53 directional selection.

54 In summary, specialized folivory is predicted to have two evolutionary effects on a
55 species' xenobiotic-metabolizing toolkit: First, genes involved in detoxification or avoidance of
56 xenobiotic toxins that are rich in a folivorous specialist's diet should show signals of selection in
57 the form of positive copy-number changes or positive selection. Second, genes *not* involved in
58 such species' defense against toxins in their specialized diet should exhibit signals of negative
59 copy-number changes and relaxation of selection. Although the first prediction is well-
60 supported by prior studies of P450 gene evolution, comparatively few studies have investigated
61 patterns of P450 gene loss or relaxation of selection in specialized folivores.
62 Bamboo lemurs are an ideal system for testing predictions about P450 gene loss in
63 specialized folivores due to their narrow diets, which are among the most specialized in the
64 entire primate order. Despite their diminutive body sizes (0.9-2.6 kg: Tan, 2006), they inhabit an
65 ecological niche that is often compared with that of the giant panda (Eronen et al., 2017;
66 McKenney et al., 2017; Tan, 2006) and are the only extant primates known to specialize so
67 intensely on bamboo or grass species (Table S1). The three sympatric species at Ranomafana
68 National Park (*i.e.*, *Hapalemur aureus, H. griseus*, and *Prolemur simus*) are the source of much
69 of the current understanding of bamboo lemur ecology. All three of these populations have
70 diets that are often predominantly composed of a small number of species of bamboo or liana
71 (Grassi, 2006; Overdorff et al., 1997; Tan, 1999), and the sympatry of these species has been
72 understood in terms of niche partitioning, notably by their differential reliance on separate
73 plant parts from the same species of giant bamboo (Glander et al., 1989). Although other
74 species outside of Ranomafana (*e.g.*, *H. meridionalis, H. alaotrensis*) do not actually consume
75 bamboo, they nonetheless eat grasses (Poaceae) or sedges (Cyperaceae) at similarly high

76 frequencies (Table S1). Thus, regardless of habitat or species, bamboo lemurs subsist on
77 constrained, highly folivorous diets.

78 One bamboo PSM is particularly intriguing for its toxicity. Consumption of the specific
79 bamboo parts consumed by *H. aureus* and *Prol. simus* exposes these lemurs to high levels of
80 potent cyanogenic glycosides (Ballhorn et al., 2009; Eppley et al., 2017; Glander et al., 1989).
81 When plant tissue is disrupted by some destructive process like mastication, these glycosides
82 will be hydrolyzed by the plant cell's own β-glycosidase enzymes to release cyanide (Ballhorn,
83 2011; Cressey & Reeve, 2019). At Ranomafana, two bamboo lemur species have been
84 estimated to consume many times the estimated lethal dose of cyanide for mammals of their
85 body sizes (Ballhorn et al., 2009; Glander et al., 1989). Although cyanide, the primary known
86 antifeedant of this bamboo, is detoxified by a metabolic pathway that does not include any
87 cytochrome P450 enzymes (reviewed by Isom et al., 2015), bamboo lemurs likely have to
88 contend with other PSMs known to be present in bamboo species, such as phenolic and
89 polyphenolic compounds (Zhu et al. 2018). Thus, we still expect evolution of bamboo lemur
90 P450 genes to be impacted by their hyper-specialized diet.

91 For this project, we hypothesized that the highly specialized diet observed for bamboo
92 lemurs should be associated with a narrower P450 repertoire, streamlined to those subfamilies
93 that metabolize xenobiotic compounds found in bamboo. Therefore, we predicted that,
94 compared to other generalist Malagasy strepsirrhines, the bamboo lemur species *Prol. simus*
95 and *H. griseus* should exhibit a clear pattern of cytochrome P450 (1) gene loss and (2)
96 signatures of relaxed natural selection, because the specialist diet contains a narrow set of
97 PSMs, of which the primary toxin (*i.e.*, cyanide), is not even metabolized by P450s. Similar to

98 previous work (Chaney et al., 2020), we focus on P450 genes in the *CYP1-3* subfamilies. Briefly,
99 this decision was made because of these subfamilies' monophyletic placement within the
100 cytochrome P450 superfamily of genes (Kirischian et al., 2011) and the presence of genes
101 encoding both xenobiotic and endobiotic metabolizers within that gene-clade (see Guengerich,
102 2015).

103 **Materials and Methods**

104 Generation of *Hapalemur griseus* genome assembly

105 For this study, we constructed a genome assembly for a male *H. griseus* (named Beavis)
106 housed at the Duke Lemur Center in Durham, North Carolina. A fresh whole blood sample was
107 shipped to the Molecular Primatology Laboratory of New York University for processing and
108 sequencing. High molecular weight (HMW) DNA was extracted from 200 µL of whole blood
109 using a MagAttract HMW DNA Kit (QIAGEN Sciences Inc., Germantown, MD) following the
110 manufacturer's protocol. DNA was eluted into a low-bind 1.5 mL tube and stored at 4°C prior
111 to size selection. In order to obtain DNA fragments roughly 40 kilobases or longer, the extracted
112 HMW DNA was run through a Blue Pippin DNA Size Selection System (Sage Science Inc., Beverly,
113 MA) using a 0.75% agarose gel cassette with the R1 size standard. Following this size selection
114 protocol, the HMW DNA concentration was quantified using a Qubit 2.0 fluorometer (Thermo
115 Fisher Scientific Inc., Carlsbad, CA) using the broad range standard. The extraction yielded 3.2
116 ng/µL of HMW DNA.

117 After extraction and quantification, the HMW DNA sample was sent to NYU Langone
118 Medical School's Genome Technology Center for quality control tests, which consisted of DNA
119 quantification and fragment size determination using a Tape Station (Agilent Technologies Inc.,

120 Santa Clara, CA). After passing this checkpoint, the HMW DNA was prepared for sequencing
121 using a Chromium Genome Linked Reads Kit (10X Genomics Inc., Pleasanton, CA) and
122 sequenced on a HiSeq 2500 DNA Sequencing Platform (Illumina Inc., San Diego, CA). The linked-
123 read output data were assembled using the Supernova assembler (Weisenfeld et al., 2017). We
124 used the pseudohaplotype output as our genome assembly for all downstream steps (Table 1).

125 Additional data gathering

126 In addition to the above-mentioned assembly for *H. griseus*, we mined data from
127 publicly available genome assemblies for 14 species: *Prolemur simus* (Hawkins et al., 2018),
128 *Lemur catta* (Palmada-Flores et al., 2022), *Eulemur flavifrons* and *E. macaco* (Meyer et al. 2015),
129 *Propithecus coquereli* (Lowe and Eddy 1997; Guevara et al. 2021), *Indri indri* (accession number:
130 GCA_004363605.1), *Daubentonia madagascariensis* (accession number: GCA_004027145.1),
131 *Mirza coquereli* (accession number: GCA_004024645.1), *Mirza zaza* (Hunnicutt et al., 2020), and
132 *Microcebus murinus* (Averdam et al., 2011; Lecompte et al., 2016), as well as the following
133 additional species of mouse lemur: *Mic. griseorufus, Mic. mittermeieri, Mic. ravelobensis,* and
134 *Mic. tavaratra* (Hunnicutt et al., 2020). These assemblies, along with any associated annotation
135 files, were downloaded locally and formatted into BLAST databases within Geneious Prime,
136 version 2022.1.1.

137 We located the loci for all annotated *CYP1-3* homologs in the *L. catta, Prop. coquereli,*
138 and *Mic. murinus* by using the associated annotation (GFF3) files for each. We defined these
139 loci by the non-P450 genes that bounded them; therefore, those surrounding genes were used
140 initially as queries for local BLAST searches. In this way, each locus was linked to two searches
141 per species. The three reference genomes listed above were used because they are all

142 members of separate strepsirrhine families (Lemuridae, Indriidae, and Cheirogaleidae,
143 respectively), and they were each therefore used as a starting point to extract the desired
144 *CYP1-3* genes or loci for confamilial species. Ideally, a pair of BLAST searches would return
145 results that included the same scaffold. By locating both BLAST hits on each of these scaffolds,
146 we were able to extract genomic regions that were hypothetically orthologous to those P450
147 loci in the *L. catta* assembly. After locating the scaffolds in each assembly corresponding to
148 each P450 locus, we used LASTZ (Harris, 2007) to interrogate the homology of those scaffolds
149 by aligning them to the confirmed P450 locus from the appropriate confamilial reference
150 genome. Positive results from these alignments were checked using the Mauve genome aligner
151 (Darling et al., 2004) on the same sequences. If output from both of these aligners indicated
152 that the reference had homology with the query scaffold(s), then the annotations from the
153 reference genome were used to extract the corresponding sequence in the other species'
154 genome. In this way, we mined the genome assemblies listed above for as many complete P450
155 genes loci as we could confidently locate.

156 <u>Inference of gene birth and death</u>

157 For this first portion of the study, we used only species for which each *CYP1-3* locus
158 could be wholly collected from a single scaffold or reasonably reconstructed if not found on a
159 single scaffold using the process described above. In order to model the events of gene birth
160 and death in this subset of lemur species, our alignment strategy followed a similar workflow as
161 outlined in previous work with other datasets (Chaney et al., 2018, 2020), but several
162 modifications were made for this project in order to allow for more standardization and
163 automation across subfamilies. First, all of the P450 genes were extracted from each species'

164 locus according to the annotation file associated with its confamilial reference. Then, all of the
165 genes from a given P450 subfamily were aligned using MAFFT (Katoh & Standley, 2013), and the
166 resulting alignment was stripped of all sites containing any gaps using trimAl (Capella-Gutierrez
167 et al., 2009). After the best-fitting nucleotide substitution model was inferred by jModelTest
168 (Darriba et al., 2012), this stripped alignment was visualized with PhyML 3.0 and the strength of
169 that resulting phylogenetic tree was tested by comparing it to 1000 bootstrap replicates
170 (Guindon et al., 2010).

171 The gene trees constructed with PhyML were then passed to Possvm (Grau-Bové &
172 Sebé-Pedrós, 2021). This program uses the intrinsic information contained in a phylogram to
173 infer speciation and gene-duplication events; it does this using the species-overlap algorithm in
174 the ETE3 toolkit (Huerta-Cepas et al., 2007, 2016). Briefly, this algorithm compares the
175 intersection of species present in both descendants of an internal node of a tree to the union of
176 species present in those descendants; using these values, the algorithm computes a species-
177 overlap score which it then uses to identify each internal node as either a speciation event,
178 having an overlap score, or a duplication event, having a high overlap score (Huerta-Cepas et
179 al., 2007). Once the identities of each node were estimated in this way, we then manually
180 examined each subtree rooted by a node called as a duplication event to infer whether any
181 gene loss had occurred. This was examined on a case-by-case basis using the reasoning that,
182 after a duplication event, each descendant of that node should recapitulate the organismal
183 phylogeny present at the time of duplication. Therefore, any species missing in one of those
184 subtrees must have lost one of the duplicates born in the earlier duplication event as long as
185 the subtree in question was well-resolved in terms of bootstrap support. In cases where

186 multiple species lineages may be absent, we deferred to the parsimonious hypothesis that a
187 loss event would have occurred prior to the divergence of those lineages, rather than a more
188 complicated hypothesis that the same paralog had been independently lost in both species
189 after their split. We visualized the Possvm output using the program Treerecs (Comte et al.,
190 2020) and then, in some cases, manually modified the depicted gene-evolution scenario in
191 order to accommodate the Possvm results.

192 Selection analysis

193 We used RELAX (Wertheim et al., 2015) to test our hypothesis concerning the relaxing of
194 selectional pressure on the P450s covered in this project. Each run of this program requires,
195 first, a codon alignment stripped of any stop codons and, second, a phylogenetic tree that
196 contains labels for the branches on which *a priori* relaxation of natural selection is hypothesized
197 to have occurred. For the codon alignments, we used preexisting annotations from each of the
198 annotated genome assemblies to extract the likely coding DNA sequence (CDS) from each of
199 the ostensibly complete P450 genes gathered in the first portion of the study. Those
200 annotations were then leveraged to find the likely CDS in unannotated homologous sequences
201 from confamilial relatives.

202 We aligned the CDS from each subfamily in Geneious Prime by translation using the
203 MAFFT aligner (Katoh & Standley, 2013), and we then checked each alignment by eye. Once the
204 alignment had been checked by eye and the maximum number of useable sequences were
205 included, it was exported in FASTA format. The trees used for all runs of RELAX were adapted
206 from the phylogenies constructed in the earlier part of this study concerned with gene birth
207 and death. We compared the rows in each subfamily's final CDS alignment to the leaves of the

208 gene tree constructed earlier with an R script using functions from the *phytools* (Revell, 2012)
209 and *seqinr* (Charif & Lobry, 2007) R packages, and a new tree was written that contained only
210 the intersection of these two sets. This filtered gene tree was then viewed with the web-based
211 *phylotree.js* platform (Shank et al., 2018), which allowed us to easily label branches on each
212 tree. For subfamilies where no duplications had been detected in the first part of this study
213 (*e.g.*, *CYP1B*), only foreground branches were labeled for *Prol. simus, H. griseus*, and any
214 internal branches exclusive to the clade formed by these two species. In these cases, all
215 unlabeled branches were used for the estimation of the background *dN*/*dS* ratios. However, we
216 followed a more complicated, yet still conservative, strategy when dealing with subfamilies
217 where multiple orthogroups were detected by Possvm (*e.g.*, *CYP2A*). For these subfamilies, all
218 non-bamboo lemur branches were marked as background branches except for the deepest
219 internal branches that separate orthogroups, which were left unlabeled. This allowed us to
220 exclude those deep portions of the phylogeny, which we imagined may have coincided with
221 instances of past neofunctionalization following gene duplication. If true, this effect would
222 probably bias the *dN*/*dS* distribution of the background branches; thus, we excluded those deep
223 branches from analysis in the relevant cases. Aside from manually reading the text-file output,
224 the HyPhy Vision platform (http://vision.hyphy.org/RELAX) was used to generate some of the
225 graphs made from the output JSON files.
226 Finally, we performed follow-up tests for diversifying selection for one gene, *CYP2F*, as
227 initial analyses with RELAX revealed unexpected signatures of selection intensification. We first
228 used BUSTED (Murrell et al., 2015) as a confirmatory check for any evidence of
229 diversifying/positive selection in the input alignment, followed by aBSREL (Smith et al., 2018), in

230 conjunction with the branch-labeling workflow described above, to test for diversifying/positive
231 selection specifically in the bamboo-lemur branches of the gene tree.

232 **Results**

233 The evidence presented here largely displays a trend in which the common lineage of
234 bamboo lemurs has a diminished or expected number of gene copies relative to generalist
235 species, with several xenobiotic-metabolizing P450 subfamilies exhibiting a resolved gene-tree
236 topology indicating genic loss in one or both of the bamboo lemur species included here.
237 Because of the other species included in this data set, there are also some insights to be gained
238 from examining other portions of the gene trees outside of the portions focused on our species
239 of interest.

240 <u>Heightened gene loss within the bamboo-lemur lineage</u>

241 The topologies of the *CYP1A* and *CYP1B* subfamilies show strong bootstrap support and
242 near-perfect correspondence to the species phylogeny for their member orthologs (Figs. S1 &
243 S2). The only exception to this derives from the *Propithecus coquereli* lineage, which saw the
244 duplication of the *CYP1A1* ortholog after the divergence of the *Prop. coquereli* and *Indri indri*
245 lineages.

246 The shape of the *CYP2A* gene tree implies substantially more genic turnover than either
247 of the *CYP1* subfamilies (Fig. 1, top-left; Fig. S3). While the *Microcebus murinus* lineage shows a
248 very straightforward trajectory of no duplications, all other species have either duplicated or
249 lost orthologs according to the pattern of relatedness displayed in this tree. Due to the mid-
250 point rooting of this tree by Possvm, it is unclear whether two or three orthogroups are truly
251 present in this case (see Fig. S3), but we opt for a parsimonious interpretation of two

252 orthogroups here (see Fig. 1). . Nonetheless, at least one duplication was certainly specific to
253 the *Prop. coquereli* lineage, depicted here by the common node of *CYP2A13L1* and *CYP2A13L2*.
254 The lemurid portion of the tree indicates a gene duplication in the common ancestor of all
255 three species and another duplication that was specific to *L. catta*. Conversely, the topology
256 displayed here implies genic loss for *E. macaco* and for *Prol. simus.*
257 The only members of *CYP2B* examined in this study are *CYP2B6* homologs because
258 another group of orthologs in the *L. catta* genome assembly (*CYP2B11*) did not show a CDS
259 length that was remotely close to the normal length of a functional primate P450 gene (*i.e.*, *ca.*
260 1500 nt). In the *CYP2B6* tree (Fig. 1, bottom-left; Fig. S4), we find two major orthogroups among
261 the five species analyzed. One of these shows a topology that, in itself, appears to recapitulate
262 the organismal phylogeny with no duplications. However, the other orthogroup exhibits a
263 signature of interesting dynamism. There are two serial duplication events in the *Prop.*
264 *coquereli* lineage (*CYP2B6L1, 3,* and *4P*). In the lemurid portion of this subtree, there are two
265 more serial duplications prior to the speciation of *L. catta* and *Prol. simus.* The pattern in this
266 part of the tree most parsimoniously indicates, first, that the bamboo-lemur common ancestor
267 lost genes orthologous to the *CYP2B6L3* and *CYP2B6L4* genes in the *L. catta* assembly and,
268 second, that the *H. griseus* lineage further lost its final remaining gene in this orthogroup.
269 The *CYP2C* genes yielded a phylogeny whose complexity is underscored by the large
270 number of duplications within it (Fig. 1, right; Fig. S5). Possvm separated this phylogeny into
271 five coherent orthogroups. The first of these (*i.e.,* OG-0) contained a duplication prior to the
272 divergence of *L. catta* and the bamboo lemurs. Following the *Prolemur-Hapalemur* divergence,
273 *Prol. simus* lost one of these genes but the other paralog was lost by *H. griseus*; thus, both

274 species retained separate paralogs. OG-1 and OG-2 lack *Prol. simus* genes but do contain genes
275 belonging to *H. griseus*; this pattern indicates two lineage specific events of gene loss for *Prol.*
276 *simus.* While this latter species, the greater bamboo lemur, showed a pattern of complete loss
277 for these orthogroups, *Mic. murinus* experienced a duplication in OG-2. All lemurid genes were
278 ostensibly lost from OG-3, while the mouse-lemur lineage saw a single-gene expansion of this
279 orthogroup. The final clade of genes, OG-4, contained three duplication events and,
280 parsimoniously, three instances of gene loss. While *L. catta* retained every one of the
281 duplicated genes from this sequence of events, bamboo lemurs likely lost the ortholog to *L.*
282 *catta CYP2C18L1*, and both *Prol. simus* and *H. griseus* further lost one more gene each.
283 However, these two final losses were lineage-specific, and each involved a separate genic
284 lineage. Thus, with the exception of genes in one orthogroup (*i.e.*, OG-3), bamboo lemurs
285 exhibited all of the losses detected in this tree.
286 Similar to *CYP2C*, the *CYP2D* subfamily also showed an increased amount of evolutionary
287 dynamism with regard to gene birth and death (Fig. 2, top-left; Fig. S6). Along a basal branch of
288 the phylogeny, there are two serial duplications, resulting in three *Mic. murinus* homologs
289 equally related to all lemurid *CYP2D* homologs. Each of the black lemur (*E. macaco*) species has
290 two very similar *CYP2D* genes which arose from the duplication specific to the *Eulemur* lineage.
291 Prior to the divergence of the *L. catta* lineage from that of the bamboo lemurs, there is some
292 uncertainty in these results whether only a single duplication occurred or whether two
293 occurred. This uncertainty stems from two nodes displaying low bootstrap support (73.0% and
294 57.5%; see Fig. S6) in the original PhyML tree. The topology displayed in Figure S6 describes two
295 duplications and parsimoniously implies just as many deletions. In light of the low bootstrap

296 support for this pattern, however, it is conceivable that the true topology could place the *L.*
297 *catta CYP2D17L2* gene into the clade with the two bamboo-lemur genes. Such a revision would
298 reduce the number of deletions and duplications. The other duplicated gene in the lemurid
299 lineage, while secondarily duplicated in the *L. catta* lineage, was lost in the *H. griseus* lineage.
300 Lastly, no functional homologs for the *CYP2D* subfamily could be retrieved for *H. griseus*
301 because no open reading frames devoid of stop codons could be found in the extracted
302 sequence for this species' single *CYP2D* gene. However, this isolated instance may be due to an
303 assembly artifact, as the *CYP2D* locus was located on the ends of two separate scaffolds in the
304 original Supernova assembly. In all, the general pattern of this subfamily is similar to that of
305 *CYP2C*: evidence of heightened genic turnover with an emphasis on loss in the specific
306 lineage(s) of the bamboo lemurs.

307 The *CYP2J* subfamily divided into three orthogroups (Fig. 2, top-right; Fig. S7). The first
308 of these (OG-0) contains only a single duplication node before the speciation node for the split
309 of *L. catta* from the bamboo lemurs. The complete lack of any bamboo-lemur genes in this
310 portion of the tree parsimoniously indicates a loss of that duplicate prior to the *Prolemur-*
311 *Hapalemur* divergence. The remaining two *CYP2J* orthogroups (OG-1 and OG-2) arose as the
312 product of a duplication event prior to the first speciation event of all species involved in this
313 analysis. While no more duplications were called in the OG1-OG2 clade, several deletions are
314 implied by the tree's topology. First, one gene appears to have been lost in the *Prop. coquereli*
315 lineage. Second, another deletion was ostensibly lost in the ancestral lemurid lineage. Last, *H.*
316 *griseus* lost one more homolog after that lineage's split from that of *Prol. simus.* Thus, the

317 *CYP2J* subfamily displays more of the above-mentioned pattern wherein the bamboo lemurs
318 stand out for their likelihood of losing P450 genes.
319 The topologies of the remaining *CYP2* subfamilies (*i.e.*, *E, F, G, R, S, U,* and *W*) show no
320 evidence of duplication (Figs. S8-S13). Degeneration of one *CYP2F* gene may have occurred in
321 the *Mic. murinus* lineage (see Fig. S9), but anthropoids and at least some lorisiforms appear to
322 possess only a single *CYP2F* gene as well (Chaney et al., 2020; Chaney, 2023). Therefore, the
323 ancestral primate number of *CYP2F* orthologs is uncertain without the inclusion of a more basal
324 species in the analysis. With this single possible exception, bamboo lemurs have clearly
325 maintained the same number of gene copies in each of these subfamilies as the other lemur
326 species analyzed here.
327 The final subfamily of this part of the analysis further underscores the pattern described
328 for other subfamilies above (Fig. 2, bottom-left; Fig. S14). While OG-0 within the *CYP3A* tree
329 possesses only speciation nodes, one loss is evident in the absence of *Prop. coquereli* from this
330 clade. The other part of the tree exhibits a very complex topology because of the number of
331 duplication nodes contained within it. Many of these outside of the lemurid portion of the tree
332 are taxon-specific: *Prop. coquereli* and *Mic. murinus* each experienced two and four exclusive
333 duplication events, respectively. The lemurid portion of OG-1 shows at least three duplication
334 events prior to the divergence of *L. catta* and from the ancestral bamboo-lemur lineage. One
335 more duplication event, leading to the *L. catta* genes *CYP3A4L1* and *CYP3A4L2*, would be most
336 parsimoniously interpreted as having occurred specifically in ring-tail lemur lineage, but it is not
337 ruled out that this duplication may have occurred in an ancestral species and been lost by both
338 bamboo lemur species. Regardless, at least two separate events of genic loss seem to have

339 occurred in the lineage of the bamboo lemurs. Thus, while *L. catta* appears to exhibit six
340 separate *CYP3A* homologs, bamboo lemurs possess half that number; furthermore, we
341 conclude that this pattern is attributable nearly wholly to gene loss by bamboo lemurs rather
342 than lineage-specific expansion of *CYP3A* for *L. catta.*

343 <u>Selection relaxed on two important P450 xenobiotic metabolizers but intensified on another</u>

344 Out of all P450 subfamilies studied here, only *CYP1A* and *CYP2D* showed significant
345 signals of selectional relaxation within the bamboo-lemur clade (Table S2). Upon comparing the
346 *dN*/*dS* distribution for the reference branches to that of the bamboo-lemur branches, it appears
347 that the signatures of relaxation in the bamboo-lemur portions of these analyses are due to the
348 lessening of the pressure for diversifying selection (Fig. 3). The degree of relaxation appears to
349 differ between these two subfamilies. RELAX's estimate for the selection intensity parameter
350 (*k*), while significantly estimated to be less than 1.00, implies only a moderate level of
351 selectional relaxation for *CYP2D*. On the other hand, in the case of *CYP1A*, RELAX's estimate
352 for *k* was especially diminutive, indicating a stronger effect for that subfamily than for *CYP2A13*
353 and *CYP2D*.

354 Interestingly, the *CYP2F* subfamily showed significant intensification of selection in the
355 RELAX models reported here, exhibiting an estimate for *k* that was significantly higher than 1.00
356 (Table S3). The *dN*/*dS* categories within this subfamily's distribution portray a subfamily under
357 purifying selection for reference branches in the model (Fig. 3). Bamboo lemurs show an
358 exaggeration of this simple pattern, first, in the elevation of the strength of signals for both
359 diversifying selection and purifying selection. We followed this RELAX analysis with analyses
360 using both BUSTED (Murrell et al., 2015) and aBSREL (Smith et al., 2018). BUSTED analyses

361 found evidence of diversifying selection within the alignment (LRT = 21.47, p = 1.1e-5), and a
362 subsequent aBSREL analysis found significant signals for diversifying selection along the
363 terminal branches for *H. griseus CYP2F2* (LRT = 9.46, p = 1.53e-2) and *Prol. simus CYP2F2* (LRT =
364 85.35, p < 1e-5).

365 **Discussion**

366 In this study, we have demonstrated that the genomes of two bamboo lemur species (*H.*
367 *griseus* and *Prol. simus*) harbor, at a conservative maximum, the modal number of cytochrome
368 P450 genes across the other lemur species included here (Table S1). Indeed, in most cases
369 where losses of P450 genes were inferred, they are most parsimoniously attributed to a
370 bamboo lemur-specific branch of the phylogeny in question (see Figs. 1-6). Thus, bamboo
371 lemurs appear to show a narrower repertoire of xenobiotic-metabolizing P450s insofar as they
372 have generally fewer genes than other species of lemur, especially in comparison to their
373 closest relative, *Lemur catta.* Bamboo lemurs also show signals of relaxation of natural selection
374 for the *CYP1A* and *CYP2D* subfamilies (see Fig. 3), which are known to be involved in xenobiotic
375 metabolism (Danielson, 2002; Guengerich, 2015).

376 These results are perhaps best understood in terms of an evolutionary tradeoff on
377 detoxification strategies (Dearing et al., 2005; Freeland & Janzen, 1974; Ueno, 2001). In a
378 pivotal paper outlining several nutritional-ecological hypotheses, Freeland and Janzen (1974)
379 formalized the notion that generalist and specialist herbivores should differ inversely with
380 regard to the breadth of their arsenals for the metabolism of toxic plant secondary metabolites.
381 In their view, herbivores should possess a comparatively wide array of detoxification
382 mechanisms to handle low doses of a commensurately broad variety of toxins, and specialist

383 herbivores should, conversely, possess a smaller array of such mechanisms able to handle large
384 doses of a narrow variety of toxins (Dearing et al., 2005; Freeland & Janzen, 1974; Marsh et al.,
385 2006). The general pattern of copy-number differences and the more limited result of
386 selectional relaxation shown in this study seems to support this hypothesis in the case of
387 bamboo lemurs, alongside this taxon's high degree of specialization (see Table S1) and the
388 stupendous amounts of cyanide consumed by them (Ballhorn et al., 2009; Glander et al., 1989).
389 Despite bamboo lemurs' observed amount of gene loss for some xenobiotic-
390 metabolizing P450 genes, the *CYP2F* subfamily contrasted with other P450 subfamilies by
391 having not only signals of selection intensification but also a clear signal of diversifying
392 selection. Although this P450 subfamily is most highly expressed in the respiratory tract,
393 hepatic expression has been demonstrated, albeit at lower levels (Duff et al., 2015; Fagerberg
394 et al., 2014; Yue et al., 2014). Based on these results, we speculated that CYP2F must
395 metabolize a PSM present in bamboo. Human CYP2F1 and its model-rodent homologs can
396 activate coumarins through epoxidation reactions, leading to cytotoxicity and necrosis at
397 varying species-dependent levels (Born et al, 2002; Vassalo et al., 2004). Similarly, CYP2F
398 enzymes have been linked with bioactivation of the toxin naphthalene, also through an
399 oxidation reaction (Lanza et al., 1999; Li et al., 2011). These biochemical associations are
400 notable here because chromatographic assays and microbiome studies of various species of
401 bamboo have implied or directly indicated the presence of both naphthalene (Li et al., 2002;
402 Takahashi et al., 2010) and coumarins (Coffie et al., 2014) in those bamboo species (reviewed
403 by Gagliano et al., 2022). Generalizing from these findings, we would hypothesize, first, that
404 naphthalene and coumarins are present in Malagasy bamboo consumed by bamboo lemurs

405 and, second, that bamboo lemurs would be susceptible to toxicity from either of these
406 compounds or their metabolite oxides, which are toxic in themselves (napthalene: see Yost et
407 al., 2021; coumarins: see Foroozesh et al., 2019). Thus, both species' *CYP2F2* orthologs may
408 have experienced selective pressure for altering its translated enzyme's affinity or efficiency for
409 either naphthalene or coumarins.

410 The difference between bamboo lemurs and the sifaka *Propithecus coquereli* stands out
411 as a second confirmatory signal of Freeland and Janzen's (1974) tradeoff hypothesis because,
412 while both of these taxa are generally classified as folivorous, they differ in their amount of
413 dietary specialization. As mentioned previously, all species of bamboo lemur that have been
414 studied are heavily reliant on either grasses (usually bamboo) or sedges, and very few plants,
415 often only a single species, are required to describe a large majority of their diets (see Table
416 S1). Conversely, sifakas (*Propithecus* spp.) consume much more varied foods (Hemingway,
417 1998; Norscia et al., 2006; Powzyk & Mowry, 2003; Rowe et al., 2016), seasonally trending
418 toward gramnivory or frugivory depending on the species in question (Irwin 2006). Despite this
419 dietary diversity, sifakas do possess clear gastrointestinal adaptations related to hindgut
420 fermentation, such as a greatly elongated intestine and an expanded, haustrated cecum
421 (Campbell et al., 2000). For this reason, sifakas are often described as *anatomical folivores*, with
422 the implication that they are more behaviorally flexible with their diet choice than one might be
423 led to believe upon viewing their gastrointestinal anatomy. Meanwhile, bamboo lemurs of all
424 three major species possess a relatively simpler gastrointestinal anatomy, with an overall
425 relatively shorter intestine and a shorter, though completely haustrated, colon (Campbell et al.

426 2000; Yamashita et al. 2008). All of this information serves to position sifakas and bamboo
427 lemurs on opposite sides of a specialist-generalist continuum among herbivores.
428 Such dietary differences between these two taxa helps to clarify an ultimate explanation
429 for the opposite pattern of gene birth and death seen in the *CYP2B6* gene cluster for bamboo
430 lemurs and *Prop. coquereli* (see Fig. 1). This is also true, but to a less pronounced degree, for
431 the *CYP2A* subfamily (see Fig. 1). In both of these cases, bamboo lemurs exhibit clade-specific
432 gene losses and *Prop. coquereli* shows an opposite signal of lineage-specific gene duplications.
433 Indeed, (Guevara et al. 2021), utilizing this same genome assembly for *Prop. coquereli,*
434 independently found that these two P450 subfamilies had been enriched in so-called "rapidly
435 evolving genes," or genes with increased *dN/dS* ratios according to PAML branch-site models
436 (Yang, 2007). Because both *CYP2A* and *CYP2B* subfamilies are involved in the metabolism of
437 xenobiotic compounds (Guengerich 2015; Orr et al. 2020), at least these two P450 subfamilies
438 stand out as candidate gene groups supporting Freeland and Janzen's (1974) prediction, that
439 specialist and generalist herbivores should differ oppositely in the diversity of their means for
440 detoxifying plant secondary metabolites. This interesting natural experiment, of comparing
441 bamboo lemurs and sifakas (or indriids in general), deserves future investigation.
442 A second line of inquiry is the contrast between *Lemur catta* and the bamboo lemurs.
443 The number of xenobiotic-metabolizing P450 genes in the *L. catta* genome was always greater
444 than or equal to those in either bamboo lemur genome. The highly varied and very seasonal
445 diet documented for *L. catta* (Jolly, 1966; LaFleur, 2012; Sauther, 1992; Sauther et al., 1999;
446 Simmen, Sauther, et al., 2006; Soma, 2006) stands out as a potential explanation for this large
447 number of detoxification mechanisms. Indeed, Sauther et al. (1999) argue that the best dietary

448 classification for *L. catta* should be “opportunistic omnivores” (p. 122) because of the breadth
449 of this species’ dietary repertoire and the drastic spikes of certain foods based on their seasonal
450 availability. This species also appears to be relatively inured to plant secondary metabolites
451 based on the high tannin and alkaloid content of their foods (Gould et al., 2009; Simmen,
452 Sauther, et al., 2006) and their relatively high rejection threshold for the bitter alkaloid quinine
453 (Simmen, Peronny, et al., 2006). This constellation of information portrays a pattern of an
454 animal that is exposed to a broad array of plant secondary metabolites and, perhaps because of
455 the annual variety of their foods, exposed to them at relatively low levels.

456 While *L. catta* possesses a sizeable number of xenobiotic-metabolizing P450 genes in
457 comparison to both species of bamboo lemur (see Table S1), these genes are not the products
458 of species-specific duplications, as is plain from the phylogenetic pattern of relatedness among
459 all the genes falling into the clade of ringtail and bamboo lemurs. Instead, many of these
460 duplicated genes seem to have arisen during the internode of a lemurid common ancestor, or
461 that of a common ancestor of the *Hapalemur* and *Lemur* genera, and then subsequently lost by
462 one or both of the bamboo lemurs species included here. This result accords well with
463 experimental work on bitter taste receptors (TAS2R16), which shows that the species of
464 bamboo lemur included here (as well as *H. aureus*) show a decreased sensitivity to several
465 bitter β-glycoside compounds (Itoigawa et al., 2021). Revealingly, however, the missense
466 mutations that most altered the response of the TAS2R16 protein were separate for *Prol. simus*
467 and *Hapalemur* spp., and ancestral reconstructions of the last common ancestors for both the
468 genus *Hapalemur* and for all bamboo lemurs showed a more sensitive response to these bitter
469 compounds than any of the present-day species-specific TAS2R16 isoforms (Itoigawa et al.,

470 2021). Given this earlier work and the pattern of P450 gene birth and death presented here, a
471 generalist dietary pattern is clearly the best estimate for an ancestral ecology of the
472 *Hapalemur-Lemur* last common ancestor. Furthermore, the complex pattern of loss and
473 retention of separate P450 paralogs by *Prol. simus* and *H. griseus* seems to agree with the
474 conclusion of Itoigawa et al. (2021) that these species' varying, but in all cases intense,
475 specialization on cyanogenic bamboo may have evolved in parallel rather than what would be
476 predicted based on parsimony, namely that this specialization would have evolved in the
477 common ancestor of all extant bamboo-lemur species.

478 On a methodological note, the pattern of gene gain and loss reported here is certainly
479 not the conclusion that would be reached by using an approach where the gene-count table is
480 parsimoniously compared to an organismal phylogeny to estimate ancestral states of gene
481 counts, as with the commonly used program for studying gene-family evolution, CAFE (De Bie
482 et al., 2006). This software program takes phylogeny into account only insofar as the species
483 themselves are concerned but does not include any information about the genes' own
484 phylogenetic relatedness, and for this reason, we see it as an undesirable alternative to
485 examining a phylogeny built from the genes themselves. While CAFE is very useful in its ability
486 to be easily automated, manual examinations of gene trees, or perhaps the use of programs
487 such as Possvm (Grau-Bové & Sebé-Pedrós, 2021) that make direct use of gene trees via the
488 species-overlap method (Huerta-Cepas, et al., 2007, 2016), would yield more accurate ancestral
489 reconstructions of gene copy-number differences among species.

490 A lingering question remains as to what compounds, or families of compounds, are
491 leading to the evolutionary expansions and contractions on display in this study. However,

492 there is currently a clear disconnect in the literature that inhibits any discussion between those
493 who have precise knowledge of P450 substrate compounds (*e.g.,* Guengerich 2015) and those
494 who perform phytochemical assays on foods of wild primates (*e.g.,* Eppley et al. 2017; Thurau
495 et al. 2021; Windley et al. 2022). The disconnect arises from differing levels of scale on which
496 each of these two groups operates: pharmacologists often use precise molecules (*e.g.*,
497 benzo[a]pyrene, aflatoxin B1) in their experiments and parlance, while phytochemists and
498 primatologists speak in the more general terms of compound families (*e.g.*, alkaloids,
499 condensed tannins). This nomenclatural gulf between these two camps is a foggy one, and it
500 serves to cloud any inferences that may be gained about the functions of free-ranging, non-
501 model primate species.

502 Here, we have found that bamboo lemurs have, on average, fewer *CYP1-3* gene copies
503 than other lemurs, and this is especially true when they are viewed alongside their closest
504 relative, the ringtail lemur. Viewed another way, bamboo lemurs have, at most, the same
505 number of P450 genes per subfamily as *L. catta*, and they often have fewer such genes. The
506 retention of these specific P450 homologs was not parsimonious when compared with the
507 organismal phylogeny, and the complexity of this pattern may imply some information about
508 bamboo-lemur evolution in general, namely that their specialization on cyanogenic bamboo
509 may have evolved in parallel rather than as a sort of ecological synapomorphy early after the
510 divergence of these two lineages. Only two P450 subfamilies (*CYP1A* and *CYP2D*), both of which
511 are linked with xenobiotic metabolism in humans (Guengerich, 2015), showed a statistical
512 decrease in the intensity of selection on the CDS for the genes within those subfamilies. As in
513 previous work on the evolution of the cytochrome P450 superfamily of genes, this topic

514 provides an interesting, complex, and sometimes unexpected window into biological evolution.
515 Future work that includes other species of bamboo lemur (*e.g.*, *H. alaotrensis, H. aureus*) will
516 doubtless only clarify that window.

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929 Data Accessibility and Benefit-sharing

930 FASTQ reads from the genomic sequencing of *Hapalemur griseus* have been deposited
931 in the NCBI SRA (accession number XXXXX), and the alignments and trees necessary to
932 reproduce this work are available on Dryad.

933 Author Contributions

934 MEC and AJT conceptualized the project. MEC collected and analyzed the data and
935 wrote the first draft of the paper, with guidance from AJT and CMB. All authors edited
936 subsequent drafts and approved of the final submission.

937 Table 1: Select statistics from de novo assembly of *Hapalemur griseus* (Beavis) using the

938 Supernova assembler.

Raw coverage	**Contig N_{50}**	**Scaffold N_{50}**	**Est. genome size**	**BUSCOs recovered (Simão et al. 2015)**
69.49x	277.73 kb	20.47 Mb	2.61 Gb[1]	3927/4104 (95.7%)

939

940 **Figure 1:** Evolution of three groups of *CYP2A, CYP2B6,* and *CYP2C* gene groups. Notes labels

941 with squares denote inferred gene-duplication events, while exes denote inferred gene losses.

942 For the sake of clarity, orthogroups are displayed as separate gene trees in this figure and color

943 coded to match orthogroup labels in Figures S3-S5. Genes labeled with asterisks are genes that

944 were originally placed in standalone orthogroups by Possvm but have been assigned to another

945 orthogroup here.

946

947 **Figure 2:** Evolution of *CYP2D, CYP2J,* and *CYP3A* gene subfamilies. Labeling conventions follow

948 those described for Figure 1.

949

950 **Figure 3:** *dN/dS*-based selection analyses of *CYP1A, CYP2D,* and *CYP2F* gene subfamilies. The

951 dashed gray lines in the bar graphs represent the points at which *dN/dS* (*i.e.,* ω) = 1.00, which is

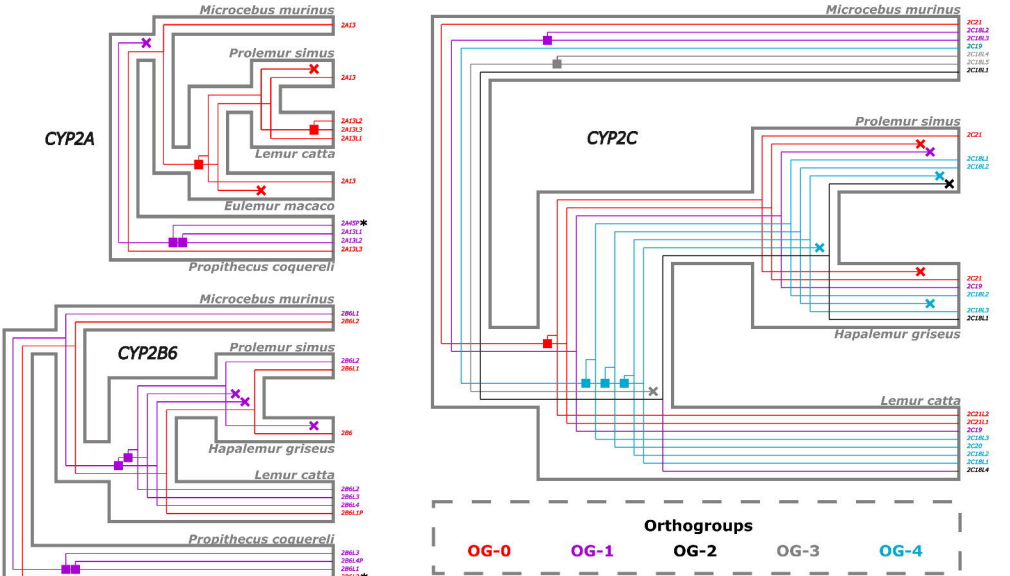
952 predicted by neutral evolution. Values less than 1.00 increasingly indicate purifying selection,

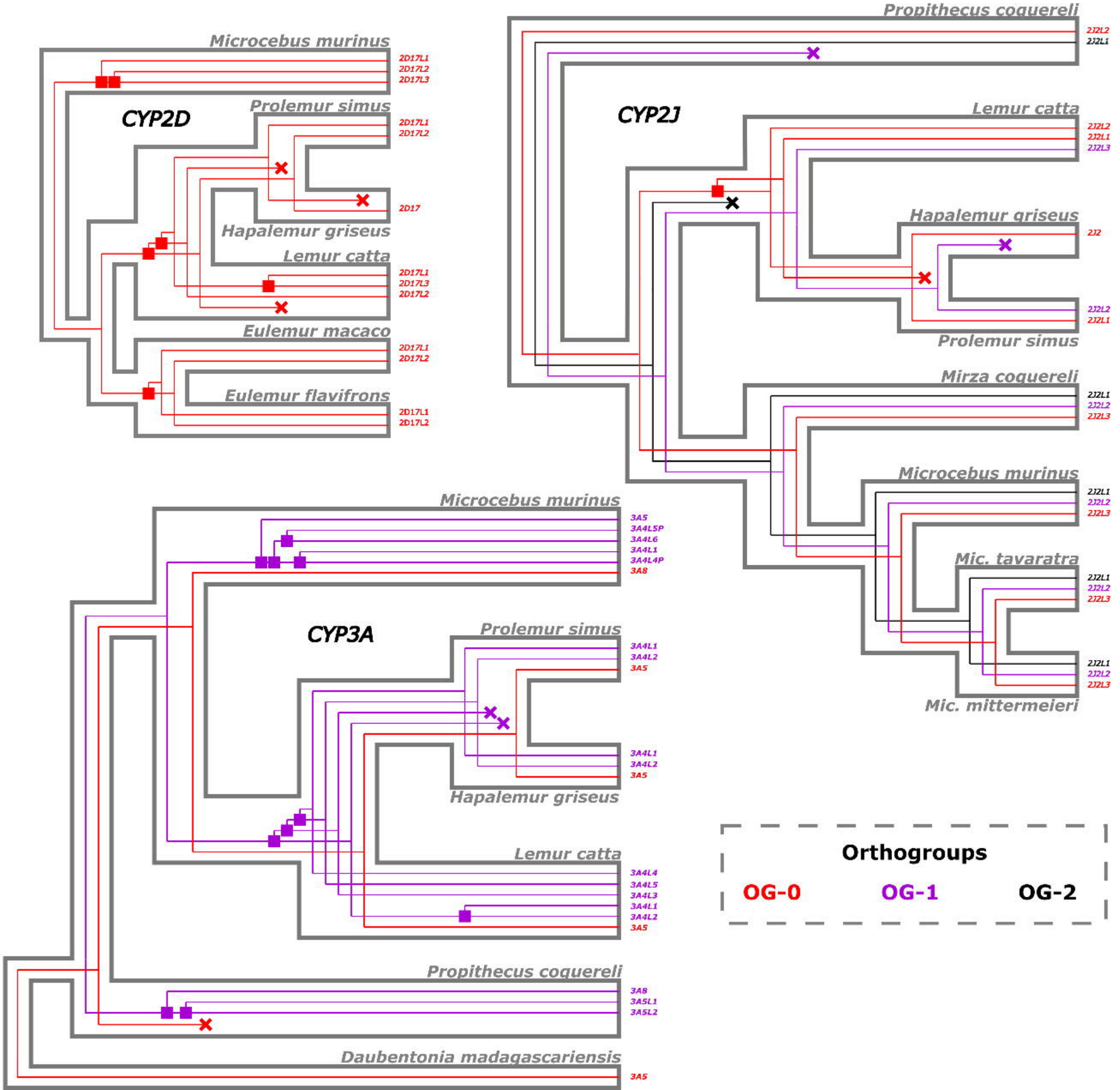
953 while ω > 1.00 indicate diversifying selection. The ω distributions of *CYP1* and *CYP2D* show

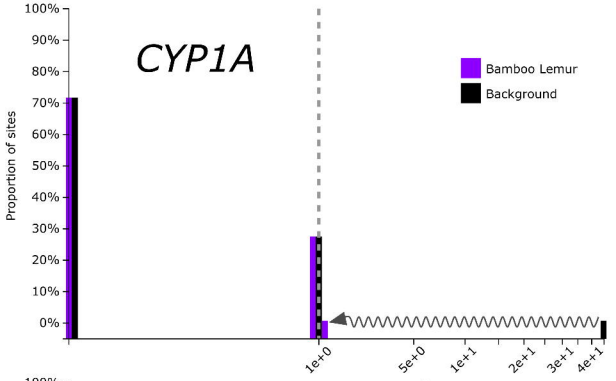
954 patterns of relaxed selection, while that for *CYP2F* indicates intensification of selection. Follow-

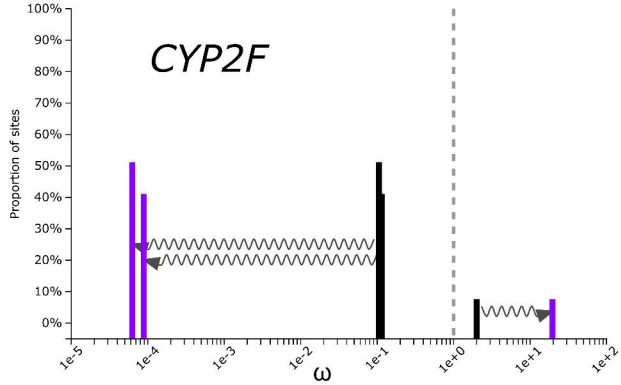
955 up HyPhy analysis using aBSREL (bottom-right quadrant) revealed a signal of diversifying

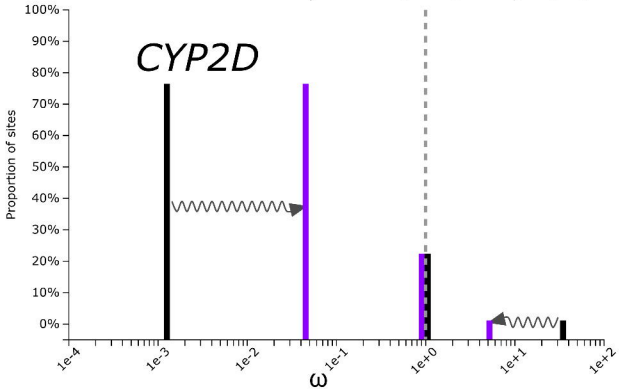
956 selection on the two terminal bamboo-lemur branches in the *CYP2F2* portion of this gene tree.

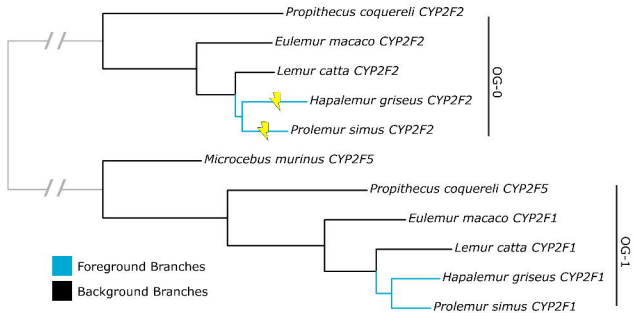

CYP2A
Microcebus murinus
2A13
Prolemur simus
2A13
2A13L2
2A13L3
2A13L1
Lemur catta
2A13
Eulemur macaco
2A459
2A13L1
2A13L2
2A13L3
Propithecus coquereli
CYP2B6
Microcebus murinus
2B6L1
2B6L2
Prolemur simus
2B6L2
2B6L1
2B6
Hapalemur griseus
Lemur catta
2B6L2
2B6L3
2B6L4
2B6L1P
Propithecus coquereli
2B6L3
2B6L4P
2B6L1
2B6L2
CYP2C
Microcebus murinus
2C21
2C18L2
2C18L3
2C19
2C18L4
2C18L5
2C18L1
Prolemur simus
2C21
2C18L1
2C18L2
2C21
2C19
2C18L2
2C18L3
2C18L1
Hapalemur griseus
Lemur catta
2C21L2
2C21L1
2C19
2C18L3
2C20
2C18L2
2C18L1
2C18L4
Orthogroups
OG-0
OG-1
OG-2
OG-3
OG-4


CYP2D
Microcebus murinus
2D17L1
2D17L2
2D17L3
Prolemur simus
2D17L1
2D17L2
2D17
Hapalemur griseus
Lemur catta
2D17L1
2D17L3
2D17L2
Eulemur macaco
2D17L1
2D17L2
Eulemur flavifrons
2D17L1
2D17L2
CYP2J
Propithecus coquereli
2J2L2
2J2L1
Lemur catta
2J2L2
2J2L1
2J2L3
Hapalemur griseus
2J2
2J2L2
2J2L1
Prolemur simus
Mirza coquereli
2J2L1
2J2L2
2J2L3
Microcebus murinus
2J2L1
2J2L2
2J2L3
Mic. tavaratra
2J2L1
2J2L2
2J2L3
2J2L1
2J2L2
2J2L3
Mic. mittermeieri
CYP3A
Microcebus murinus
3A5
3A4L5P
3A4L6
3A4L1
3A4L4P
3A8
Prolemur simus
3A4L1
3A4L2
3A5
3A4L1
3A4L2
3A5
Hapalemur griseus
Lemur catta
3A4L4
3A4L5
3A4L3
3A4L1
3A4L2
3A5
Propithecus coquereli
3A8
3A5L1
3A5L2
Daubentonia madagascariensis
3A5
Orthogroups
OG-0
OG-1
OG-2


CYP1A
Bamboo Lemur
Background
Proportion of sites
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1e+0
5e+0
1e+1
2e+1
3e+1
4e+1


CYP2F
Proportion of sites
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1e-5
1e-4
1e-3
1e-2
1e-1
1e+0
1e+1
1e+2
ω


CYP2D
Proportion of sites
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1e-4
1e-3
1e-2
1e-1
1e+0
1e+1
1e+2
ω


Propithecus coquereli CYP2F2
Eulemur macaco CYP2F2
Lemur catta CYP2F2
Hapalemur griseus CYP2F2
Prolemur simus CYP2F2
OG-0
Microcebus murinus CYP2F5
Propithecus coquereli CYP2F5
Eulemur macaco CYP2F1
Lemur catta CYP2F1
Hapalemur griseus CYP2F1
Prolemur simus CYP2F1
OG-1
Foreground Branches
Background Branches