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Many options, few solutions: over 60 million years snakes converged on a few optimal venom formulations

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21 Abstract

22 Gene expression changes contribute to complex trait variations in both individuals and
23 populations. However, how gene expression influences changes of complex traits over
24 macroevolutionary timescales remains poorly understood. Being comprised of proteinaceous
25 cocktails, snake venoms are unique in that the expression of each toxin can be quantified and
26 mapped to a distinct genomic locus and traced for millions of years. Using a phylogenetic
27 generalized linear mixed model, we analysed expression data of toxin genes from 52 snake
28 species spanning the three venomous snake families, and estimated phylogenetic covariance,
29 which acts as a measure of evolutionary constraint. We find that evolution of toxin
30 combinations is not constrained. However, while all combinations are in principle possible, the
31 actual dimensionality of phylomorphic space is low, with envenomation strategies focused
32 around only four major toxins: metalloproteases, three-finger toxins, serine proteases, and
33 phospholipases A2. While most extant snakes prioritize either a single or a combination of
34 major toxins, they are repeatedly recruited and lost. We find that over macroevolutionary
35 timescales the venom phenotypes were not shaped by phylogenetic constraints, which include
36 important microevolutionary constraints such as epistasis and pleiotropy, but more likely by
37 ecological filtering that permits a few optimal solutions. As a result, phenotypic optima were
38 repeatedly attained by distantly related species. These results indicate that venoms evolve by
39 selection on biochemistry of prey envenomation, which permit diversity though parallelism and
40 impose strong limits, since only a few of the theoretically possible strategies seem to work well
41 and are observed in extant snakes.

42
43 Keywords: gene expression, generalized linear mixed model, macroevolution, parallel
44 evolution, venom

45

46

47 Introduction

48 Single genes underlying major traits are the exception rather than the rule, and the dissection
49 of polygenic trait variation has been at the forefront of biological research (1–3). Much of the
50 complexity resulting from interactions between genes is mediated through their expression,
51 which plays a central role in determining phenotypic variation between individuals and
52 populations (4–8). In particular, levels of gene expression account for substantial sources of
53 variation in natural populations, acting as potential targets of natural selection (8–10).
54 Although population-level differences in expression may contribute to the onset of local
55 adaptation and perhaps even eventual adaptive divergence (6, 11, 12), how changes in gene
56 expression levels lead to evolution of complex traits over the course of millions of years
57 remains largely unknown.

58
59 Interactions between genes and their effect in channelling of adaptive responses have been
60 the focus of the field of quantitative genetics. How evolution results from the combined effects
61 of the adaptive landscape, and the pattern of genetic variances and covariance among genes
62 (the G matrix), is one of the key questions in this field (13, 14). The covariance between genes
63 plays a vital role in shaping complex traits by determining the evolutionary trajectory through
64 natural selection (15), and the occurrence of parallelism (16). While most quantitative genetics
65 studies deal with populations, their conclusions can translate to macroevolutionary processes
66 as well. For example, estimates of divergence between populations show that the direction of
67 greatest phenotypic divergence can be predicted by the multivariate direction of greatest
68 additive genetic variance within populations (17). Unfortunately, the G matrix cannot be
69 extrapolated across macroevolutionary timescales, as it itself evolves (18). Fortunately, it is
70 possible to compute a phylogenetic covariance matrix for multivariate traits, which can serve
71 as a useful analogy to the G matrix, but over much larger timescales, and incorporating a
72 broader range of constraints (19, 20). We can then examine whether the structure of the
73 phylogenetic covariance matrix corresponds to evolutionary trajectories of complex traits.

74

75
76 Here we use the analogy between the G matrix and the phylogenetic covariance matrix to
77 understand how gene expression evolves in a complex trait, namely snake venom. Being
78 composed of proteinaceous cocktails, snake venoms are unique in that the expression of each
79 toxin type can be quantified and traced to a distinct genomic locus (21–23). Variations in gene
80 expression alter the abundance of proteins in the venom, thereby influencing venom efficacy
81 (24–26). Thus, toxin expression levels constitute the polygenic phenotype that is the venom,
82 allowing us to examine how selection affects gene expression over tens of millions of years.
83 To examine the features of complex trait evolution at the level of gene expression, we
84 estimated phylogenetic covariance of 10 toxins using data from 52 snake species covering
85 the three venomous snake families (Elapidae, Viperidae, and Colubridae) and asked the
86 extent to which it matched observed patterns of evolutionary change across taxa.

87
88
89 Although we find that extant snake venoms occupy a limited area of phenotypic space, largely
90 centred around four major toxin families, there are no phylogenetic constraints to the number
91 of possible venom combinations. These data show that the relatively small number of
92 molecular strategies used by the snakes result from consistent and often convergent selection
93 on the biochemistry of envenomation, rather than from intrinsic constraints on gene
94 interactions. Thus, over tens of millions of years selection likely plays a greater role in shaping
95 the venom phenotype than intrinsic constraints.

96 Results

97 **Expression data and phylogeny**

98 Expression data for snakes were collected from published studies that reported relative levels
99 of toxin expression via next-generation (Illumina and 454) transcriptome sequencing of cDNA
100 libraries. We obtained data for a total of 52 different snake species from the three major

101 venomous families (Colubridae, Elapidae and Viperidae), from a list of 39 publications
102 (Supplementary Table 1). For inclusion, each study had to provide quantitative data on toxin
103 component abundance and had species for which phylogenetic data were available. We
104 restricted our dataset to include components that are found in at least 50% of snakes to focus
105 on generally important toxins, and because sample sizes for the other components would be
106 too low for accurate and phylogenetically unbiased inference. Overall 10 out of 27 toxins we
107 retained. For comparative analyses, we used a published time-calibrated phylogeny of
108 squamates, which estimated the most recent common ancestor (root) of the three snake
109 families to about 60 million years ago (27).

110

111 **Evolutionary covariance between venom components**

112 By limiting the range of responses to natural selection, the covariances between genes reflects
113 constraints that shape a phenotype. The phylogenetic covariance matrix (PCOV) accounts for
114 the effect of phylogeny on the interrelationships between genes coding for the snake venom
115 phenotype, providing an approximation of the presence or absence of constraint behind the
116 evolution of gene expression levels. To estimate the PCOV, we used a phylogenetic
117 generalized linear mixed model (PGLMM) under a Bayesian framework. The concept of
118 PGLMM was devised in the early 90s as a method to infer evolutionary constraints of
119 characters using only phylogeny and measures of phenotypes (19). As an extension of
120 maximum likelihood based techniques widely used in quantitative genetics, PGLMM was
121 notable for its versatility as a comparative method (28, 29). We use a modern rendition of the
122 PGLMM devised by Hadfield and Nakagawa, which was optimized for faster and better
123 performance (29, 30). The PGLMM estimated changes in gene expression against a
124 presumed change in diet with the effect of phylogeny being modelled as a random effect. Life
125 history characteristics and diets for snakes are difficult to obtain, particularly in a consistent
126 manner. However, a snake's potential diet is largely affected by its body size, with smaller
127 individuals consuming smaller prey, while larger individuals tend to prefer larger prey (31).

128 Therefore, we used adult snake length as a proxy for diet. The mean effective sample size for
129 all parameters was greater than 11,000 (Supplementary figure 4). The diagnostics revealed
130 suitable convergence of the chains with negligible autocorrelation in the MCMC
131 (Supplementary Fig. 1-3). Significant values in the PCOV matrix denote the presence of
132 phylogenetic constraint, while non-significant values denote its absence. We observed a lack
133 of significant values in the PCOV (Fig. 1) for all the venom components that we modelled. In
134 addition to estimating a PCOV, the model was used to compute λ values which denote the
135 phylogenetic signal (Fig. 1), similar to Pagel's λ model for phylogenetic signal (29). The λ
136 values are a measure of statistical dependence of trait values and phylogeny. They indicate
137 whether certain components in modern snakes were likely similar as in their ancestors. In our
138 case, most venom components show strong phylogenetic signals of greater than 0.5, albeit
139 with large confidence intervals. However, and all venom components have λ significantly
140 greater than zero. A few, in particular cysteine-rich secretory proteins (CRISPs),
141 metalloproteinase (SVMP), three finger toxin (TFTx), and Kunitz-type serine protease inhibitor
142 (KSPI) show very strong phylogenetic signals (> 0.8) and narrow confidence intervals,
143 indicating the presence of strong phylogenetic inertia.

144

145 **Four toxins drive the evolution of the snake venom arsenal**

146 We identified axes of maximum variations in the toxin components using PCA on the
147 phylogenetic covariances, using it to visualize the dimensionality of the venom phenotype (32).
148 The first two components, which jointly explained 73.6% of the variation, had the largest
149 loadings from four families of toxins: three finger toxins (TFTx), snake venom
150 metalloproteinase (SVMP), phospholipase A₂ (PLA₂), and snake venom serine protease
151 (SVSP) (Fig. 2). We therefore classified them as 'major' toxins, representing three largely
152 distinct envenomation strategies focussed around SVMP, TFTx, and a combination of PLA₂
153 and SVSP.

154 The clustering of snakes on this phylomorphic venom space shows a clear association
155 between family and the major component in the venom. For example, most elapids venoms
156 form a cluster dominated by TFTx, which is the principal family found in their venom. On the
157 other hand, vipers occupy a larger region of phylomorphospace because some have venoms
158 dominated by SVMP, while others use different combinations of SVMP, SVSP and PLA2.
159 Finally, colubrid venoms are the most diverse in composition, employing all the of the different
160 strategies. A key observation in the PCA is that some distantly related species cluster together
161 around the same envenomation strategy, suggesting parallel evolution.

162 It is important to note that PLA2s in elapids (group I) and vipers (group II) are produced by
163 different loci and have apparently evolved independently (33, 34). In order to account for any
164 underlying family-specific evolutionary trend, we conducted a parallel analysis by splitting
165 PLA2 into elapid PLA2 (ePLA2) and viperid PLA2 (vPLA2) (Supplementary Fig. 5). This
166 analysis produced qualitatively the same results as the combined analysis, though the first
167 two components of the PCA explained less variance (61.8% as opposed to 73.6%). In
168 particular, loadings for both elapid and viperid PLA2 were oriented in the same direction
169 (Supplementary Fig. 10), consistent with the previous observations that they convergently
170 evolved similar toxic activities (35). Thus, we carried out all subsequent analysis by combining
171 them into a joint functional category.

172

173 **Parallelism of envenomation strategies**

174 The clustering of distantly related species in the PCA despite the generally high phylogenetic
175 inertia hinted at the likely parallelism of envenomation strategies across snakes. To test for
176 parallelism across the phylogeny we used SURFACE (36), which fits a series of stabilizing
177 selection models to identify instances where multiple lineages adopt the same selective
178 regime (36). SURFACE uses AIC as criterion to determine goodness of fit, and keeps adding
179 models until the AIC doesn't improve further (36). The final model included 11 regime shifts
180 and 4 distinct regimes ($\Delta k = 4$) and a $c = 7$ convergent shifts. The AIC improved from 572.5

181 to 438.25 in the forward phase, to a final AIC of 407.56 in the backward phase (S11) which
182 indicated that the final model was a better fit than the initial ones. The SURFACE model
183 revealed widespread convergence in elapids, vipers, and colubrids (Fig. 3). Vipers showed
184 evidence of three distinct regimes, out of which two evolved in parallel (Fig. 3, Supplementary
185 12). One of the convergent regimes focused on SVMP evolved repeatedly in viperids and
186 colubrids (Fig. 3). Another strategy, adopted by three species across all three families
187 (*Ovophis okinavensis*, *Crotalus simus*, and *Pseudonaja textilis*) was focussed around SVSP
188 (Fig. 3). In elapids, there was greater evidence for a single convergent regime focused around
189 TFTx. We used the inbuilt simulation function in SURFACE to obtain a null distribution on a
190 simulated dataset using a Hansen model that lacked true convergence (36, 37). Comparison
191 to the null model simulations (Supplementary Table 2) revealed significantly more convergent
192 regimes obtained from our analysis than would be obtained by chance ($p=0.030$). This allowed
193 us to reject the null hypothesis and state that the cases of convergence are due to some
194 optima in the phenotypic adaptive landscape.

195

196 **Strategies based on major components evolved at different times**

197 Understanding the ancestral state of a trait can paint a picture of the journey taken by the trait
198 through evolution. We used ancestral state reconstruction (ASR) analysis to estimate
199 recruitment times of the major venom components into the venom arsenal, and how venoms
200 have changed throughout the course of evolution. Because of the diversity and plasticity of
201 the venom phenotype, confidence intervals at the root were very large, and the inference of
202 the venom in the most recent common ancestor should be interpreted with caution, particularly
203 concerning absence of individual toxins. Of the four major components that are responsible
204 for venom diversification, the ASR detected only SVMP in the most common ancestor of the
205 snakes (~60 million years ago, hence forth referred to as ‘the ancestral venom’) (Fig 3). The
206 ASR reveals SVMP to be a major and widespread component for most of the evolutionary

207 history of snakes. However, at the base of elapid radiation, SVMP was largely replaced by
208 TFTx as the major component in elapid venoms. TFTx was likely present prior to the split of
209 colubrids and elapids, but while elapids have focused primarily on TFTx, colubrids employed
210 a combination of TFTx and SVMP throughout their evolution. In vipers SVMP has taken
211 various paths, from being the predominant component in Viperinae (*Echis and Bitis*), to
212 diversifying substantially in the Crotaline clade (*Protobothrops, Bothrops, Crotalus*, etc). The
213 ASR suggests that high levels of PLA2 and SVSP (which is mostly restricted to vipers) are
214 more recent additions to the venom. Although not shown in our analysis, PLA2 (both group I
215 and group II) was most likely present at the common ancestors of both Elapids and Crotalids
216 (34), but became substantial parts of the venom from around 20 million years ago in both
217 these taxa as observed from their increased occurrence. While we had estimated ancestral
218 states for the other 6 components as well (Supplementary Fig. 23-34), we limited our
219 discussion to only the major toxins since they dominate adaptive optima in the venom
220 phylomorphospace.

221

222 Discussion

223 We set out to understand how changes in gene expression underlie the evolution of a complex
224 trait, the snake venom. First, we examined the dimensionality of this trait by estimating
225 phylogenetic covariances between expression levels of individual toxins. The covariances
226 between toxin expression levels can be viewed as constraints that limit the evolution of a trait,
227 analogously to the G-matrix in quantitative genetics. Unlike the G-matrix, which arises largely
228 from pleiotropic interactions between genes, phylogenetic constraints may additionally include
229 ecological, developmental, physiological, and other factors. Significant covariance between
230 individual components would reflect constraints on evolutionary change and the total

231 phenotypic space attainable by selection (38). Thus, traits that are constituted by genes under
232 high constraint would not be able to diversify as freely as traits with no constraint. Genetic
233 constraints also determine convergence and parallel evolution, where high constraint reduces
234 the likelihood of genes contributing to different convergent regimes (16). Yet, for snake venom
235 genes we see no such constraints in gene expression, suggesting that all toxin combinations,
236 in principle, are possible (Fig. 1).

237
238 While the lack of constraint between components implies that venom has the potential to
239 diversify freely and fully fill the possible phenotype space, this is far from what we observe.
240 Rather, the total phenotypic space has surprisingly low dimensionality, with two principal
241 components explaining 73% of the variance. Venoms form three distinct clusters around the
242 major toxin components in the phylomorphospace, indicating the possible presence of three
243 distinct adaptive optima (Fig. 2). Similar toxin-specific strategies have been observed between
244 populations of snakes, but we show that the trend extends phylogenetically to different species
245 as well as different families (39, 40). While individual venom components do exhibit significant
246 phylogenetic inertia (Fig. 1), the phylomorphospace clusters often include unrelated taxa,
247 suggesting shifts in envenomation strategies between adaptive optima. These shifts likely
248 result from parallelism, which may be facilitated by lack of constraints between components
249 (Fig. 3).

250
251 Is this lack of constraint surprising for a trait like snake venom? To answer this we need to
252 understand one of the key processes by which novel functions and variations in gene families
253 arise – gene duplication (41–44). Gene duplication can cause functional redundancy by
254 producing gene copies where the original gene carries out its designated function while the
255 new copy has no active role in the biological process, thus freeing it from selective constraints
256 (41, 45, 46). This relaxed selective constraint could allow the duplicated genes to diversify
257 freely, as long as one of the copies performs the essential function, and the presence or
258 absence of another copy does not affect fitness. Therefore, a system that comprises of many
259 duplicated gene families would also likely have the ability to diversify freely. Snake venom fits

260 this characteristic since it consists of gene families that have undergone varying degrees of
261 duplications throughout their history (47). We hypothesize that the lack of constraint observed
262 between expression levels of genes encoding for snake venom could be due to the fact that
263 snake venom comprises of duplicated genes.

264
265 One of the most prevalent theories about the origins of venom composition suggests that they
266 originated after ancestral physiological genes underwent duplication and neofunctionalization
267 (48). Since venom phenotypes need to be flexible and to adapt quickly, duplicated genes
268 make ideal toxin candidates as they are under lower selective constraints (49–51). In addition
269 to sequence-level changes, changes in gene expression also contribute to microevolution in
270 snake venom (52). To get a complete picture of the evolution of the snake venom phenotype,
271 we need to understand how microevolution (changes in gene expression over short time
272 scales) relates to macroevolution (selection over large time scales). From our observations,
273 we propose a model for snake venom evolution that could potentially link the two, and explain
274 why in spite of having the potential to freely evolve, snake venom has such low dimensionality.
275 We propose that gene duplication facilitated recruitment of physiological genes into the venom
276 system, following which expression levels were free to respond to natural selection due to
277 their low constraint and to potentially occupy a wide phenotypic space. The venom
278 compositions that provided the greatest adaptive advantage due to their favourable
279 biochemistry of envenomation is what we see in present-day species. These observed
280 adaptive optima are dominated by the four main toxins leading to a high degree of parallelism.
281 This model could likely explain why snake venom, like other systems comprising of duplicated
282 genes, experience both positive and relaxed purifying selection (23, 53).

283 Temporal patterns in venom evolution

284 Ancestral snake venom composition has received considerable attention, but until now the
285 analyses have been qualitative in nature (39). While the confidence intervals for ancestral
286 state reconstruction (ASR) are large (Supplementary Fig. 14-34) owing to the remarkable

287 evolutionary lability of venom, we can nonetheless make a number of observations about the
288 course of evolution of major components. Among the major components, the ancestral venom
289 most likely contained only appreciable amount of SVMP (Fig. 3). This finding is consistent with
290 previous estimates of a likely recruitment of SVMP into the venom at the split of vipers and
291 colubrids (~62 million years ago) (24, 54). Furthermore, the SVMP-focused strategy is the only
292 convergent selective regime identified by the SURFACE analysis in all three families (Fig. 3),
293 suggesting that the machinery to produce this toxin exists in all of them. While we could not
294 detect PLA2, TFTx, and SVSP with confidence in the most recent common ancestor, they
295 could have been present at lower levels in the ancestral venom, or as ancestral precursor
296 molecules (33, 34, 55). This is especially likely given that all three families have some level of
297 each of the major toxin classes (Fig. 3).

298 Being present in the ancestral venom, SVMP continued to be used as a major toxin by viperids
299 and is still the dominant toxin in some genera (*Echis and Bitis*), as well as some colubrids.
300 However, other toxins were recruited (or increased in quantity) later in venomous snake
301 evolution. For example, consistent with previous work that placed recruitment of TFTx before
302 the divergence of modern elapids (56), we also show that TFTx was likely present prior to the
303 split between elapids and colubrids. At that time TFTx may have co-occurred with SVMP prior
304 to the split of Elapids and Colubrids, perhaps as a specific strategy, one that is quite rare in
305 present-day snakes, being found only in the colubrid brown tree snake (*Boiga irregularis*), and
306 to an extent in the king cobra (*Ophiophagus hannah*). With the proliferation of the TFTx family
307 elapids have largely lost their reliance on SVMPs.

308 Viperid and elapid sub-families have convergently evolved greater reliance on PLA2 toxins
309 (group I in elapids and group II in viperids), but have diverged in venom phenospace due to
310 the previous co-option of different major components (TFTx for elapids, SVSP for vipers). The
311 likely presence of PLA2 (group II) gene copies at the common ancestors of Crotalids raises
312 questions about when the complex expanded in the course of snake evolution (34). From our
313 analysis, we believe that the expansion started somewhere around 20-25 million years ago in

314 vipers, and was already established as a substantial part of the venom before the split of
315 *Crotalus*, and *Protobothrops* genera. In elapids ASR does not detect the use of PLA2 before
316 its recruitment as a major component of coral snakes (*Micrurus*) about 20 million years ago,
317 but it was likely present at the common ancestor of elapids and maybe even colubrids because
318 of its presence in many extant species. Interestingly, the recruitment of the two PLA2 families
319 by elapids and viperids occurred at roughly the same time, perhaps as a result of convergent
320 selection driven by radiations in prey lineages, such as mammals.

321 The overall trend is that recruitment of major toxins took place at different times, and has
322 progressed along different trajectories in different lineages, with instances of both loss and
323 heightened expression. Snakes have then shifted focus on specific toxin families, occasionally
324 investing into new toxin categories for their arsenals (e.g., PLA2s and SVSPs). The increased
325 concentration of specific venom components, relative to the ancestors, has most likely
326 happened by increases in copy number of the specific gene families (47, 48, 52). Interestingly,
327 shifts in selective regimes produced parallel specialization on the same toxin family by
328 different snakes (Fig. 3), suggesting that at the level of toxin family selection generally favours
329 specialization as opposed to diversity.

330 Conclusion

331 The extent to which traits are constrained by their history, vs reaching their fitness optima has
332 been a major debate in evolutionary biology. Numerous studies have relied on phylogenetic
333 regression to estimate morphological covariation between traits while accounting for
334 phylogenetic non-independence (20, 57–60). In our approach we analyse more than one
335 response variable simultaneously and incorporate effects on trait relationships that arise
336 through shared ancestry (61). We show that the structure of the gene expression PCOV can
337 give insights into how traits evolve, by providing a conceptual bridge between micro and
338 macroevolutionary forces. By showing that the phenotypic space is inherently unconstrained,

339 we are able to highlight the existence of fitness optima, and explain the existence of
340 widespread parallelism seen in snake venoms. These findings show that in the long-term
341 snakes are able to overcome the inherent trade-off between fitness and phylogenetic
342 constraints. Once genes underlying more traits are known in other systems, subsequent
343 studies will show to what extent snake venoms are typical of the general evolutionary pattern.

344
345

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350 several additional data sets. Nick Friedman from the Biodiversity and Biocomplexity unit at
351 OIST for discussions regarding comparative methods.

352

353

354 Author contributions

355 Dataset was collected by AB. Both AB and ASM analysed the data. AB and ASM wrote the
356 paper.

357

358 Additional information

359 Supplementary information, including code, data, original figures are available at:

360 <https://agneeshbarua.github.io/Many-options-supplementary/>

361

362 **Materials and Methods:**

363 **Data collection**

364 Toxin expression data was collected from 39 publications (online supp), while mean size
365 measurements were obtained from Encyclopedia of life and The Reptile Database (62, 63).
366 Out of the vast repertoire of venom toxins we selected only 10 as they were the most reported
367 toxins amongst all snakes. Toxins levels were recorded as per publication. Toxin values
368 reported as absolute FPKM values, were converted to a percentage of the total. All analyses
369 were carried out using this curated dataset. The toxin values were normalised for calculating
370 the PCOV and in SURFACE analyses. Measurements of snake size (total length, average
371 length, snout-vent length) as reported in the online databases (62, 63) was used in the analysis.
372 If the length was reported as a range, midpoint value was recorded in the dataset.

373
374

375 **Phylogenetic tree**

376 We used a time-calibrated tree of squamate reptiles (snakes and lizards) based on two large
377 datasets comprising of 44 nuclear genes for 161 squamates, and a dataset of 12 genes from
378 4161 squamate species, both these datasets represented families and subfamilies (27, 64,
379 65). The result was an extensive phylogeny of squamates both in terms of sampling of genes
380 and species. Fossil based age constraints were used in time-calibrating the tree making it
381 ideal for studies of biogeography, diversification and trait evolution (27). All analysis was
382 carried out using a pruned version of this tree (Supplementary Fig. 14) that contained the 52-
383 snake species for which we collected gene expression data. This pruned tree had a time at
384 root estimated to be approximately 60 million years ago.

385

386 **Estimating Phylogenetic covariance matrix**

387 The effect of phylogeny was modelled using the method stated in section 11.2.1- “General
388 Quantitative Genetic Methods for Comparative Biology” (29). Analysis was carried using the
389 MCMCglmm package in R (61). The model was written based on the description given in
390 section 3 on the MCMCglmm vignette for modelling multi-response traits (61). Phylogenetic
391 generalised linear mixed models allow for testing slightly complicated models, provide more
392 than a simple qualitative estimate of the existence of phylogenetic structure, and have greater
393 statistical power than typically used metric randomization approaches (66). The MCMC was
394 run for a total of 20 million iterations, with burnin and thinning values of 500,000 and 1,500
395 respectively. Diagnostics for the MCMC run were done by obtaining the plot for the MCMC
396 and autocorrelation. The phylogenetic signal was obtained by dividing the covariance for each
397 toxin by the total covariance of the toxin and the residuals, as mentioned in (29). We performed
398 principal components analysis using the phylogenetic covariances obtained from the
399 MCMCglmm analysis. Species codes are provided in supplementary note 1.

400 Convergence analysis

401 We used the default Ornstein-Uhlenbeck process, a convenient representation of evolution
402 towards adaptive peaks for modelling convergence in the SURFACE analysis (36). The
403 SURFACE method uses Hansen’s approach (Hansen model) of modelling evolution towards
404 different adaptive optima by painting multiple adaptive hypothesis onto branches of a
405 phylogenetic tree(36, 67). SURFACE is unique because unlike previous methods that utilize
406 Hansen models, the placements of regime shifts is guided by trait data as opposed to some a
407 priori hypothesis regarding the location of convergence (36). The SURFACE method is divided
408 into two phases. The forwards phase adds successive regimes to a basic Hansen model using
409 input from continuous trait measurements, which in our study were normalized measurements
410 of gene expression for the four major toxins. The performance of each successive model was
411 measured using AIC by balancing improvements in log-likelihood against increase in model
412 complexity (36). Since AIC for the models are calculated after adding log-likelihoods, the AIC
413 for successive models may improve. The regime shift representing the best model is painted

414 onto the tree. The backwards phase is the second phase in the analysis. During this phase of
415 SURFACE all subsets of regimes are collapsed to yield distinct regimes. The collapse is
416 continued till the AIC of the models does not increase further. The final model has k regime
417 shifts, and k' distinct regimes, in addition to the extent of convergence which is defined as the
418 difference of these terms (Δk), c is used to represent the the shifts towards different
419 convergent regimes in multiple lineages (36). We used all standard parameters as mentioned
420 in the SURFACE vignette (37). To obtain a null distribution we ran 500 iterations of the in-built
421 *surfaceSimulate* function using a Hansen-fit model, and concatenated the output from each
422 iteration.

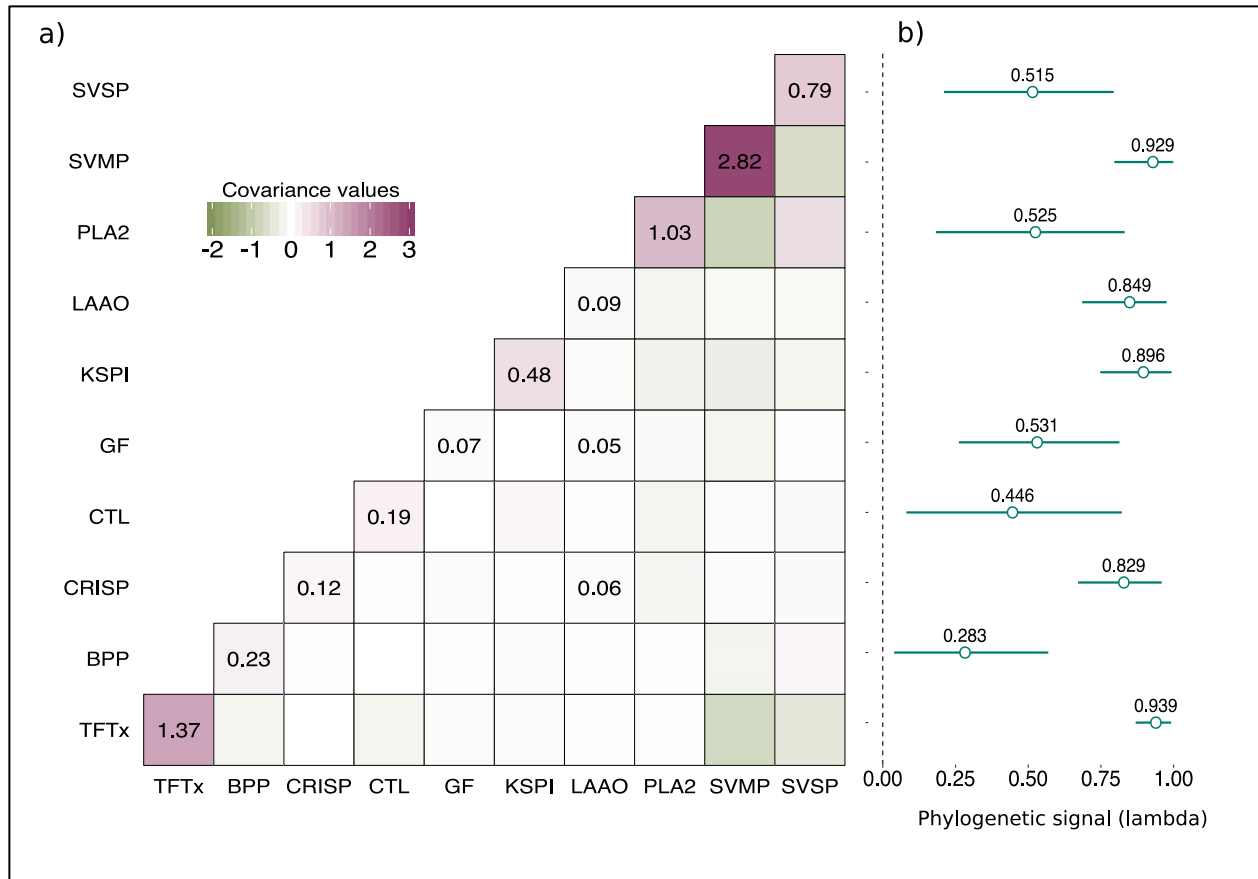
423

424 Ancestral state reconstruction (ASR)

425 The default parameters for the *fastAnc* function implemented in the Phytools package was
426 used to perform the ASR (68). A phenogram, which shows relative positions of species in
427 evolutionary phenospace, was plotted for each toxin using a spread cost of 0.1
428 (Supplementary Fig. 15-34). We used the contMap function in Phytools to obtain a tree for
429 changing trait values on a continuous scale represented by a color spectrum. Confidence
430 intervals were plotted on the nodes as bars. Only traits whose confidence intervals did not
431 overlap zero (only positive values) were considered to be present at the root. Pie charts in the
432 main figure were drawn by calculating the relative levels of each of the major toxins estimated
433 by the ASR at the specific node. Two images in the main were obtained from Wikimedia under
434 the creative commons license ([Elapidae](#): Thomas Jaehnel, [Colubridae](#): Carlo Catoni) image
435 for Viperidae provided by Alexander S. Mikheyev)

436

437 **Figures:**



438
439

Fig. 1. Phylogenetic constraints on individual toxins and their combinations. a, A lack

440

of significant values (only significant values labelled) in the phylogenetic covariance matrix

441

denote a lack of phylogenetic constraint between toxins. **b,** Components show a significant

442

presence of a phylogenetic signal, indicating that closer species are likely to evolve the same

443

way. Lambda, represents phylogenetic signal, which is a measure of dependency of trait

444

evolution with phylogeny. Lambda values, are estimated as toxin variance on the diagonal,

445

divided by the sum of diagonal variance and residuals. TFTx, SVMP, KSPI, LAAO, and CRISP

446

showed the highest signal, with greatest significance, while the rest showed comparatively

447

weaker signals. Phylogenetic constraints determine convergence and parallel evolution,

448

where high constraint reduces the likelihood of genes contributing to different convergent

449

regimes (16). Yet, for snake venom genes we see no such constraints in gene expression

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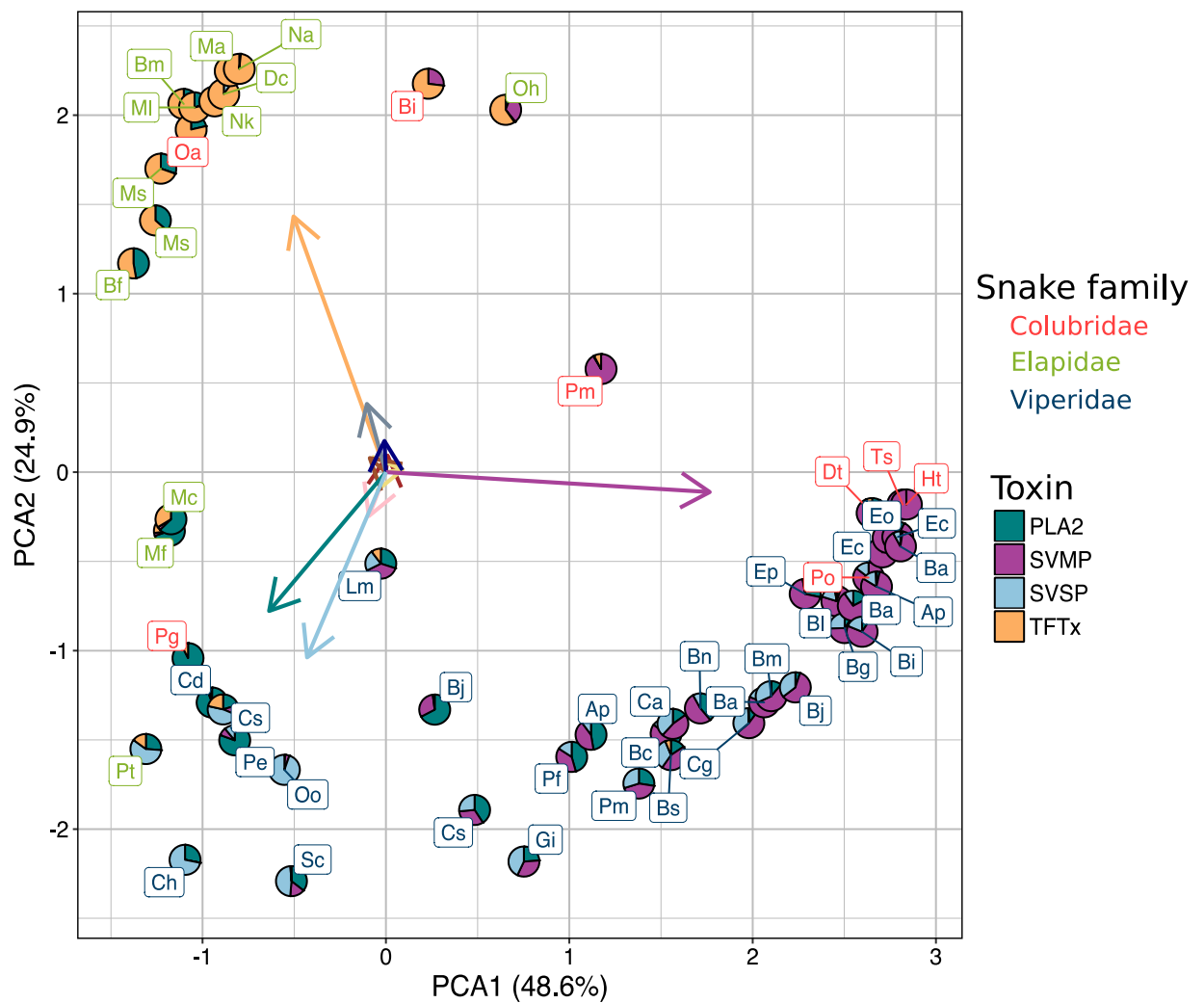
despite the high phylogenetic signal, suggesting that all toxin combinations, in principle, are

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possible.

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 455 **Fig. 2: Snakes cluster on phylomorphospace along the axes of four toxins:**
 456 Phospholipase A2 (PLA2), Snake venom serine proteases (SVSP), snake venom
 457 metalloproteases (SVMP), and three finger toxins (TFTx), (Species codes in Supplementary
 458 Note 1). These axes represent three distinct envenomation strategies employed by the snakes.
 459 Vipers in our data employ a wide spectrum of strategies, from being focused primarily on
 460 SVMP, to employing a mixture of PLA2 and SVSP. Most elapids in our data employ a strategy
 461 primarily based on TFTx, while two *Micurus* species (Mc, Mf) have a combination of PLA2
 462 and TFTx. Colubrids show a unique trend of being scattered throughout the
 463 phylomorphospace, having at least one species adopting each of the three strategies. Despite
 464 the lack of constraint in gene expression, the snake venom phenotype has very low
 465 dimensionality with the four major components accounting for 73.5% of the variation.

466 Clustering of distantly related snakes to around a similar strategy hint at the likely parallelism
467 of these major toxins.

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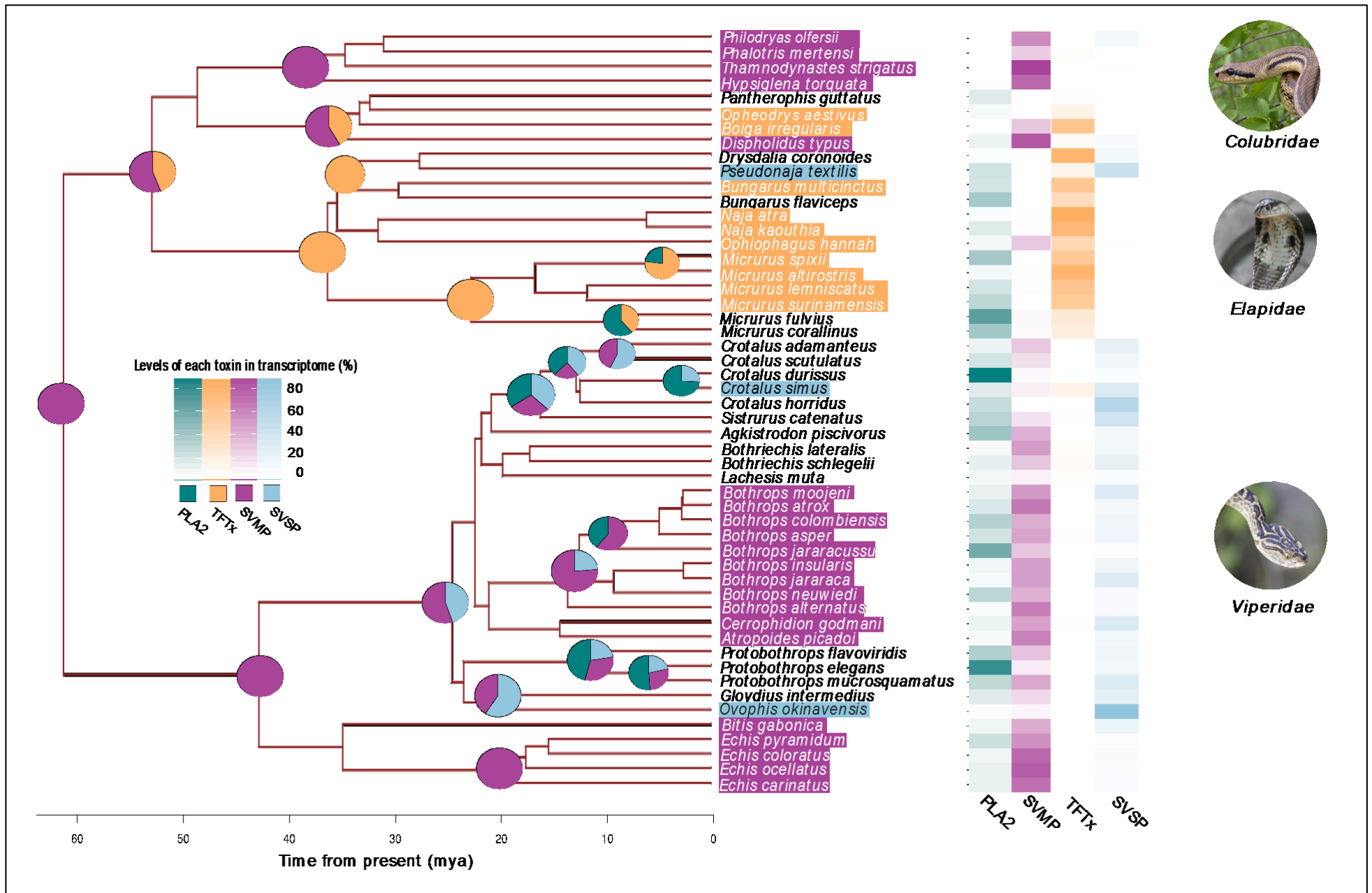


Fig. 3: Ancestral venom (at the root 60 million years ago) was likely unspecified, and among the major components contained only SVMP. The specialization of snake venom occurred relatively recently, in the past 20-40 million years, as denoted by the ancestral state reconstruction along the nodes. Different species specialized using similar components leading to a high degree of parallelism (common selective regimes are indicated by highlighted species names). Tiles represents the relative abundance of venom toxin in extant snakes. Although ancestral states were reconstructed at each node, for clarity only the ones where substantial changes in toxin levels took place are labelled. The overall trend is that starting from an undifferentiated ancestor, snakes have increasingly focused on specific toxin families, occasionally investing into new toxin categories for their arsenals (e.g., PLA2s and SVSPs). The increased concentration of specific venom components, relative to the ancestors, has most likely happened by increases in copy number of the specific gene families.

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