



HAL
open science

Unexpected colour pattern variation in mimetic frogs: implication for the diversification of warning signals in the genus *Ranitomeya*

Ugo Lориoux-Chevalier, Mario Tuanama Valles, Stephanie Gallusser, Ronald
Mori Pezo, Mathieu Chouteau

► To cite this version:

Ugo Lориoux-Chevalier, Mario Tuanama Valles, Stephanie Gallusser, Ronald Mori Pezo, Mathieu Chouteau. Unexpected colour pattern variation in mimetic frogs: implication for the diversification of warning signals in the genus *Ranitomeya*. Royal Society Open Science, 2023, 10 (6), 10.1098/rsos.230354 . hal-04215782

HAL Id: hal-04215782

<https://hal.science/hal-04215782>

Submitted on 22 Sep 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Title:** Unexpected colour pattern variation in mimetic frogs: implication for the diversification of
2 warning signals in the genus *Ranitomeya*.

3 **Authors line:** LORIOUX-CHEVALIER, Ugo¹; TUANAMA VALLES, Mario²; GALLUSSER,
4 Stephanie²; MORI PEZO, Ronald²; CHOUREAU, Mathieu¹.

5 **Affiliations:**

6 ¹ LEEISA, UAR 3456, Université de Guyane, CNRS, IFREMER, Cayenne, GF

7 ² Instituto de Investigación Biológica de las Cordilleras Orientales, Tarapoto, PE

8 **Corresponding Author:** LORIOUX-CHEVALIER Ugo, ugo.lorioux-chevalier@cnrs.fr

9 **Keywords:** Müllerian mimicry, Aposematism, Standing variation, Evolution, Radiation, Phenotype.

10 **Acknowledgements :**

11 We would like to thank Marco León and Henri Delgado from the Peruvian INIBICO NGO for the
12 constant support and logistical help provided during the realization of this project. This project would
13 not have been possible without the knowledge and frog catching abilities of Josh Allen, Cesar Lopez,
14 Manuel Panaifo, Mark Pepper, Manuel Sanchez and Evan Twomey. We would like to thank Vincent
15 Debat, Marianne Elias, Violaine Llaurens, Melanie McClure and Alexandre Roland for their
16 constructive comments on the manuscript. This research was authorized by the Peruvian Servicio
17 Nacional Forestal Y De Fauna Silvestre – SERFOR (RDG N° D000442-2021-MIDAGRI-SERFOR-
18 DGGSPFFS and 232-2016- SERFOR/DGGSPFFS).

19 **Competing interests:**

20 No competing interests

21 **Funding:**

22 This research was supported by the French National Agency for Research (ANR) grant RANAPOSA
23 (ref. ANR-20-CE02-0003) and from an “Investissement d’Avenir” grant CEBA (ref. ANR-10-LABX-
24 25-01) to MC, and from a doctoral grant « contrat doctoraux handicap » from the Ministère de
25 l’enseignement supérieur, de la recherche et de l’innovation to ULC.

26

27

28

29

30

31 ***Abstract:***

32 Predation is expected to promote uniformity in the warning colouration of defended prey, but
33 also mimicry convergence between aposematic species. Despite selection constraining both colour-
34 pattern and population divergence, many aposematic animals display numerous geographically
35 structured populations with distinct warning signal. Here, we explore the extent of phenotypic
36 variation of sympatric species of *Ranitomeya* poison frogs and test for theoretical expectations on
37 variation and convergence in mimetic signals. We demonstrate that both warning signal and mimetic
38 convergence are highly variable and are negatively correlated: some localities display high variability
39 and no mimicry while in others the phenotype is fixed and mimicry is perfect. Moreover, variation in
40 warning signals is always present within localities, and in many cases this variation overlaps between
41 populations, such that variation is continuous. Finally, we show that coloration is consistently the least
42 variable element and is likely of greater importance for predator avoidance compared to patterning.
43 We discuss the implications of our results in the context of warning signal diversification and suggest
44 that, like many other locally adapted traits, a combination of standing genetic variation and founding
45 effect might be sufficient to enable divergence in colour pattern.

46 ***Introduction***

47 Phenotypic variation between individuals is the fuel for evolution, as natural selection operates
48 by favoring individuals with traits better suited to the local environment (1). Because geographical
49 differences in the biotic (e.g. predation, competition, parasitism) and/or abiotic (e.g. salinity, sunshine,
50 temperature) environment can occur, divergent selection can favor distinct phenotypes in different
51 localities (2). The level of standing genetic variation within a species then determines whether a
52 phenotype that is advantageous in a given environment can be produced, thereby facilitating the
53 colonization of this new environment by this species.

54 Warning signals displayed in defended prey (i.e. aposematism) are adaptive traits expected to
55 be locally monomorphic (3,4). The learned avoidance of these signals by predators then results in
56 positive frequency dependent selection: the most common phenotype is favoured. This selection thus
57 promotes the persistence of a single warning signal within a population and the evolutionary
58 convergence of warning signals among both coexisting defended (Müllerian mimicry) (5) and
59 palatable species (Batesian mimicry) (6). Because the ensuing stabilizing selection is expected to
60 quickly eliminate any individual with a novel (e.g. mutants, recombinant) or exotic warning signal
61 (e.g. migrants), it should effectively limit the amount of variation present in that trait, thus strongly
62 preventing the diversification of warning signals. Yet, while local monomorphism is generally
63 observed within defended species, those species often display a geographic mosaic of distinct
64 aposematic “races” or ecotypes throughout their range (7). For example, up to 25 distinctive
65 geographic races of the chemically defended butterfly *Heliconus erato* have been documented

66 throughout south America (8,9), while the strawberry poison dart frog, *Oophaga pumilio*, displays up
67 to a dozen geographic morphs in Costa Rica and Panama (10). The same pattern is observed in
68 *Apheloria* millipedes, with 9 geographic ecotypes, found within a small region of the Appalachian
69 mountains (11), and in the nudibranch *Goniobanchus splendidus*, where 5 distinct geographic morphs
70 have been documented along the eastern coast of Australia (12). These examples, spanning a broad
71 taxonomic scale, highlight the extensive geographic variation in warning signals.

72 Both empirical and theoretical studies suggest that this mosaic-like aposematic landscape can
73 be generated by positive frequency dependent selection acting on a randomly distributed diversity of
74 aposematic signals (13). Such evolutionary process, once it lead to the fixation of different warning
75 signals in distinct localities, is maintained by its resulting geographic structure of predator that have
76 learned to avoid the local warning signals (4,13–18). Nevertheless, other selection pressures, that can
77 occur independently or in synergy with predation, may also contribute to spatially structured colour
78 pattern variations. For example, spatial heterogeneity in thermal selection can favor different melanic
79 morphotypes, as in the tiger moth *Arctia plantaginis* (19). Furthermore, assortative mating based on
80 colour patterns may reinforce barriers to gene flow between races, as in *Heliconius himera* and
81 *H.erato* where males are more likely to approach females displaying their own warning pattern (20).
82 Finally, geographic variations in aposematic communities can result in warning signal diversification
83 when the prevalence of defended model varies spatially within the range of a mimetic species (13,14).
84 This phenomenon has been documented in beetles (21), millipedes (11), and dart frogs (22,23).

85 While the persistence of spatially structured variation in warning pattern has been extensively
86 studied, identifying the mechanisms underlying the initial diversification of warning signals remains
87 challenging. Indeed, decreased phenotypic variability as a result of predators selecting against rare
88 phenotypes should impede the diversification of warning signals. Different hypotheses have therefore
89 been proposed to explain the initial process of this diversification of warning signals, including
90 shifting balance (24–26), whereby genetic drift, novel mutations and recombination, under relaxed
91 predation pressure, generates the variation that enable phenotypic evolution from one adaptive peak to
92 another. Decreased selection pressure associated with increased warning signal variability has been
93 documented in the mimetic poison frog *Ranitomeya imitator* (24), in the dyeing poison frog
94 *Dendrobates tinctorius* (27), and in the tiger moth *A. plantaginis* (28). Another possible mechanism of
95 diversification is adaptive introgression, whereby gene flow results in the incorporation of a foreign
96 aposematic variant in the species' gene pool, resulting in an important phenotypic leap and potentially
97 providing an immediate local mimetic advantage (29–31). Another possibility is that predators may
98 associate specific colour pattern elements to defences independently of the rest of the warning signal
99 (such as, for instance, a yellow spot or band; or colour vs pattern) (12,32). Predators may generalise
100 learned avoidance to all individuals with this pattern element, regardless of the rest of the phenotype,
101 thus allowing phenotypic divergence (7,33). All these different hypotheses of warning signal

102 diversification nevertheless rely on the maintenance, even transient, of trait variability within
103 populations.

104 While the expectation for warning signals is the fixation of a single phenotype and mimicry
105 convergence between aposematic species sharing morphological similarities and predators, few studies
106 have documented the extent to which warning signals truly vary within natural populations.
107 Theoretical models of selection provide a conceptual framework to predict how the intensity of
108 selection can shape both warning signal variation and mimetic relationships if these two phenomena
109 are driven by the same selection: an increase in the intensity of stabilizing selection by predators is
110 expected to lead to warning signal uniformity and promote warning signal convergence between
111 unrelated species (mimicry), while a decrease in predation will result in an increase of the warning
112 signal variation (i.e. due to stochastic neutral evolution and/or demographic process counteracting the
113 effect of selection), and mimicry is expected to be absent or imperfect. Therefore, differences in the
114 intensity of predations are expected to generate a negative relationship between the amplitude of
115 variation in warning signals within populations and the degree of mimicry between sympatric species.
116 Yet, if warning signal variation and mimicry convergence are not driven by the same selection, the
117 described negative relationship is not expected to exist.

118 The Peruvian poison dart frogs, *R. variabilis*, *R. imitator* and species of the *R. fantastica*
119 clade (consisting of the closely related *R. fantastica*, *R. summersi* and *R. benedicta*) provide an ideal
120 biological system to investigate how phenotypic variability in warning signals can be maintained, and
121 how it relates to mimetic relationships. These sympatric species are known for their impressive degree
122 of warning signal ecotypism and for their Müllerian mimetic relationship (34); indeed, only a few
123 kilometers often separate completely distinct warning forms within a single species (hereafter referred
124 to as ecotypes), and the local warning signal is often shared with the other *Ranitomeya* species living
125 in sympatry. This spatial mosaic of mimetic convergence suggests that the different species of
126 *Ranitomeya* are targeted by the same predator communities, generating locally selected warning
127 phenotypes. Interestingly, previous work on *R. imitator* has located two geographic regions with high
128 phenotypic variability known to be transition zones (24,35). In one of those regions, polymorphism
129 was facilitated by decreased local selection pressure (24).

130 In this study, we empirically test the negative relationship between local warning signal
131 variation within a given species and the degree of mimetic convergence between species within natural
132 populations of mimetic *Ranitomeya* poison dart frogs. To test for gradual vs. punctuational evolution
133 of colour pattern, we then precisely describe the geographic phenotypic landscape to assess whether
134 warning signals of the different populations (i.e. ecotypes) are characterized either by discrete or
135 continuous phenotypic variation. Continuous phenotypic variation between locally common ecotypes
136 would suggest that standing variation within the metapopulation favours geographic diversification of

137 colour patterns. Finally, we dissected the different components of the warning signals to determine if
138 certain colours or pattern elements are less variable than others, concordant with stronger stabilizing
139 selection on these characteristics than on the rest of the phenotype.

140

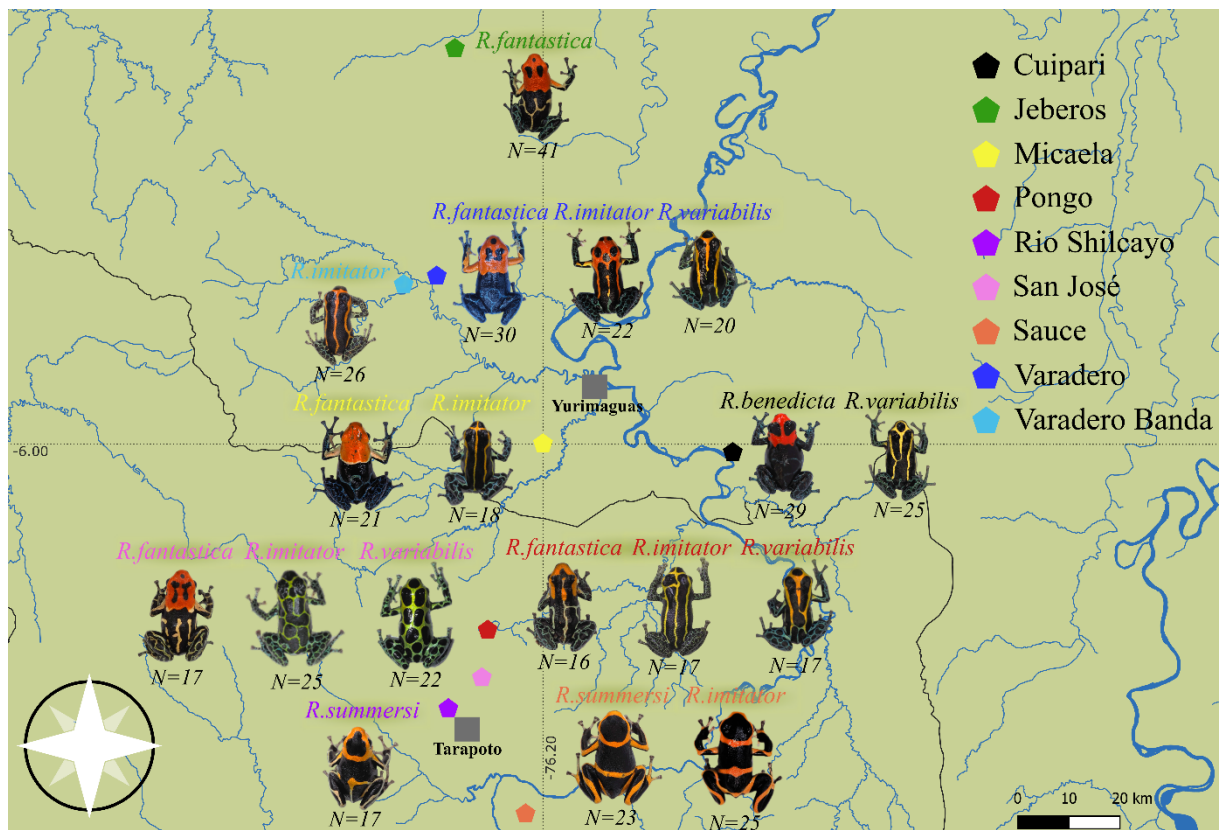
141

142

143 **Materials and methods**

144 *Sampling*

145 *Ranitomeya variabilis*, *R. imitator* and the species of the *R. fantastica* clade (*R. fantastica*, *R.*
146 *summersi* and *R. benedicta*) are amphibians of the *Dendrobatidae* family. Like all species of the genus
147 *Ranitomeya*, these species possess vivid colour patterns and chemical defences (potent skin alkaloids)
148 that deter predators (36). In the studied region of Northern Peru (San Martin and Loreto department),
149 these species have a different number of ecotypes: 2 mimetic ecotypes have been described for *R.*
150 *variabilis*, 4 mimetic ecotypes for *R. imitator*, and up to 9 ecotypes for the *R. fantastica* clade (34,35),
151 each characterized by a distinct warning signal. To quantify phenotypic variation within these different
152 ecotypes of *Ranitomeya*, we sampled frogs from 9 localities that differed in the warning signal:
153 Cuipari, Jeberos, Micaela, Pongo, Rio Shilcayo, San-José, Sauce, Varadero and Varadero Banda
154 (suppTab.1, Fig.1).



156

157 **Figure 1: Distribution map of the sampling sites and the associated local ecotypes for all studied**
 158 **species in the region of San Martín and Loreto, Northern Peru.**

159 At least 2 species live in sympatry in 7 of the 9 localities. In each locality, all mature frogs
 160 encountered were captured and immediately photographed back at the lab under standardized
 161 conditions using a tripod-supported Olympus EOS 5D Mark III camera with an Olympus 60mm macro
 162 lens. A X-rite™ ColourChecker Passport Photo 2 was used in every picture to get an accurate
 163 correction for luminosity and colours between frogs. Four hundred and eleven *Ranitomeya* frogs of the
 164 3 species (i.e. 195 *R. fantastica* clade, 132 *R. imitator* and 84 *R. variabilis*) were photographed.

165 *Colour and pattern quantification*

166 Quantifying the warning signal is challenging due to the multiple pattern elements and colours
 167 that needs to be analysed simultaneously to capture the whole visual signal. Here we used a
 168 combination of methods to capture the different types of colour and pattern variation. First, frog
 169 pictures were standardized for colours using the X-rite™ colour standard and the X-rite™ 2019
 170 ColorChecker Camera Calibration V1.1.3 software. We then divided the frog pictures into three body
 171 parts: head, dorsum and hind legs, as we generally observe a transition of colours and patterns between
 172 these parts. For each 3 body parts, we quantified both the colour and the shape of the non-black
 173 elements, resulting in a total of 6 phenotypic characteristics.

174 To quantify the colour of the warning signal, we used the Hue, Saturation, Brightness (HSB)
175 domain (37) using the software Photoshop™. Using the magic wand tool in Photoshop CC 2019, we
176 selected the area of interest and extracted the mean HSB value of the body part. The brightness (B)
177 value was then removed from the HSB value as this consists of the luminance present where the frogs
178 were photographed, which fluctuates depending on the amount of light present. Furthermore, although
179 poison frogs are attacked by a variety of predators (snakes, spiders, ants...) (38), birds which are able
180 to recognise colour across Hue and Saturation under diverse conditions of brightness (39) are believed
181 to be the major agent of selection on these frogs' aposematic signal (40,41).

182 To quantify the pattern, we used three different methods. We first used the R package
183 Patternize (42) to characterize the head pattern. The head pattern was analysed using the pixel-pixel
184 homology comparison of stacked images implemented in Patternize. Prior to using Patternize, 10
185 landmarks were therefore positioned on all frog pictures using imageJ™ enabling the precise stacking
186 of the patterning elements (suppFig.1). To limit the analysis to only the head, the head pattern was
187 manually coloured in red using Gimp, and Patternize was instructed to only extract and analyse this
188 colour. Warning signal pattern elements of the back and the legs range from wide transversal bands to
189 fine reticulation. While the developmental process controlling this patterning is unknown in
190 amphibians, they are reminiscent of the processes of reaction-diffusion patterning (43). Because this
191 process has a certain randomness, the patterns produced are not adequately analyzed by Patternize (i.e.
192 the position of the reticulations of two equally reticulated frogs will always be different) and this
193 results in similarly reticulated frogs being considered as very different. Hindlimbs and dorsal patterns
194 were thus quantified using the "adjacent method" (44). This method is based on transitions between
195 colour patches, which amounts to describing the amount of reticulation (the alternation of black
196 background and colour pattern). To implement this method for quantifying the dorsal pattern, two
197 parallel transects on each side of the spine, from the neck to the groin on the back, were sampled.
198 Along these transects we counted the number of colour changes, effectively quantifying the degree of
199 reticulation; i.e. from a simple lateral line to a complex network of reticulation. Another transect going
200 from the groin to the knee was used to quantify the reticulation of the legs. As the front and hindlimbs
201 share a similar pattern and colour, only the hindlimbs were used.

202 Finally, we also used an additional method to characterize the pattern using qualitative
203 categories, as it has been shown that the pattern of the different *Ranitomeya* ecotypes can be
204 successfully classified as either striped (anteroposterior lines), spotted (reticulated patterning), lined
205 (dorsotransversal patterning band) or diffuse (uniform colour pattern on the head and random pattern
206 on the back (35)). We use this qualitative grouping method based on the four patterning groups to
207 correct for the high variation found within some ecotypes, and that may result in similar frogs being
208 identified as quantitatively very different (e.g. the mimetic spotted morphs of *R. variabilis* and *R.*
209 *imitator* both show high levels of variation in spot number).

210 To analyse the warning phenotype and compare it between individuals, each of the matrices
 211 for the 7 warning signal characteristics measured (i.e. head, body and limb pattern, head, body and
 212 limb colour + qualitative pattern) were transformed into a normalized symmetrical Euclidian distance
 213 matrix for quantitative measures, and into a symmetrical distance matrix for the qualitative variable
 214 using Dice distance. Each phenotypic matrix (*mat*) was then normalized using the following formula
 215 where *min* and *max* represent respectively the lower and higher value of the matrix.

$$NM = \frac{mat - \min}{max - \min}$$

$$Matglob = \sqrt{(NMcolour.head^2 + NMcolour.back^2 + NMcolour.limb^2 + NMpattern.head^2 + NMpattern.back^2 + NMpattern.limb^2 + NMclassif.pattern^2)}$$

216 These 7 normalized matrices (*NM*) were subsequently squared, summed and a square root
 217 transformation was applied to obtain an overall distance matrix (*Matglob*), effectively giving all 7
 218 characteristics identical weight.

219 ***Relationship between warning signal variability and the degree of mimicry convergence***

220 Differences in the intensity of predator selection have been documented within certain localities of the
 221 studied *Ranitomeya* (24). With increasing predation selection for warning signal uniformity, we expect
 222 to observe a decrease in signal variation and an increase in mimetic resemblance between *Ranitomeya*
 223 species. We tested this theoretical expectation of a negative relationship between warning signal
 224 variability and mimicry resemblance. To quantify phenotypic variation of the warning signal for each
 225 of the populations, we calculated the mean pairwise phenotypic distance between individuals within
 226 each population (*Var*). To assess the degree of convergence (from perfect mimics to incomplete or
 227 ongoing mimetic convergence), we estimated whether the population of interest is phenotypically
 228 more similar (or different) to the other sympatric species than it is from allopatric populations of
 229 conspecifics. We calculated the distance using the population centroids. Dividing the phenotypic
 230 distance from its sympatric species by the mean distance from allopatric populations of conspecifics
 231 gave us the mimetic distance value. We then divided one by the sum of *Im* to transform this distance
 232 value to a measure of mimicry similarity (*ms*); this results in a value ranging from 0 to $+\infty$, with values
 233 increasing with more perfect mimicry and values below 1 indicating no mimicry convergence is
 234 detected. This metric informs us of the amplitude of the divergence from conspecific a population
 235 underwent due to mimicry. Finally, after testing the normality of this distribution with a Shapiro-Wilk
 236 normality test ($W \geq 0.920$, $P \geq 0.1966$), we used a linear Pearson correlation to test for the negative
 237 relationship between warning signal variation and the degree of mimicry convergence.

238 ***Exploring phenotypic variability within and between ecotypes***

239 To investigate whether phenotypic distribution is continuous or discrete between distinct
240 ecotypes of each species, we plotted the morphospace of the quantitative measures of the warning
241 signal using Multidimensional Scaling. Warning signals are under positive frequency dependent
242 selection, which means that their fitness increases with their abundance. Plotting each individual
243 phenotype enables us to visually ascertain the distribution of distinct density peaks which correspond
244 to abundant warning signals. Theoretically, these phenotypic peaks should coincide with fitness
245 optima separated by low fitness valleys. As it includes all localities, this visual representation (i.e.
246 morphospace) depicts how the fitness associated to warning signals is geographically structured. The
247 exploration of the shape (height, variability and distance between them) of the distinct peaks within
248 the morphospace highlights the possible effect of geographic differences in natural selection in
249 constraining, or not, the warning signals of each population. This also enables us to ascertain if the
250 phenotypic distribution is discrete or continuous between the different ecotypes.

251 *Variation within colour and/or pattern elements vs whole phenotypes*

252 To assess whether some characteristics of the warning signal are less variable than others,
253 possibly as a result of stronger stabilizing selection, we compared the phenotypic variability for each
254 of the 6 quantitatively measured characteristics of the warning signal within each locality and species.
255 The phenotypic variability was obtained by calculating the mean phenotypic distance between every
256 combination of two individuals of the same population for a given phenotypic characteristic. We also
257 computed the sum of the variance for each characteristic among all populations. A Kruskal-Wallis test
258 followed by a Wilcoxon test with Bonferroni correction was performed to assess whether some
259 phenotypic characteristics of the warning signal were more variable than others within each ecotype.
260 Similarly, we also tested whether comparable levels of variation are observed for a given phenotypic
261 characteristic between sympatric species. This prediction is expected if predator selection acts with the
262 same intensity and direction on the warning signal in different sympatric *Ranitomeya* species. Finally,
263 the comparison performed between all localities for all three species can inform on whether some
264 colour pattern characteristics are under stronger selection, suggesting they are important for predator
265 recognition.

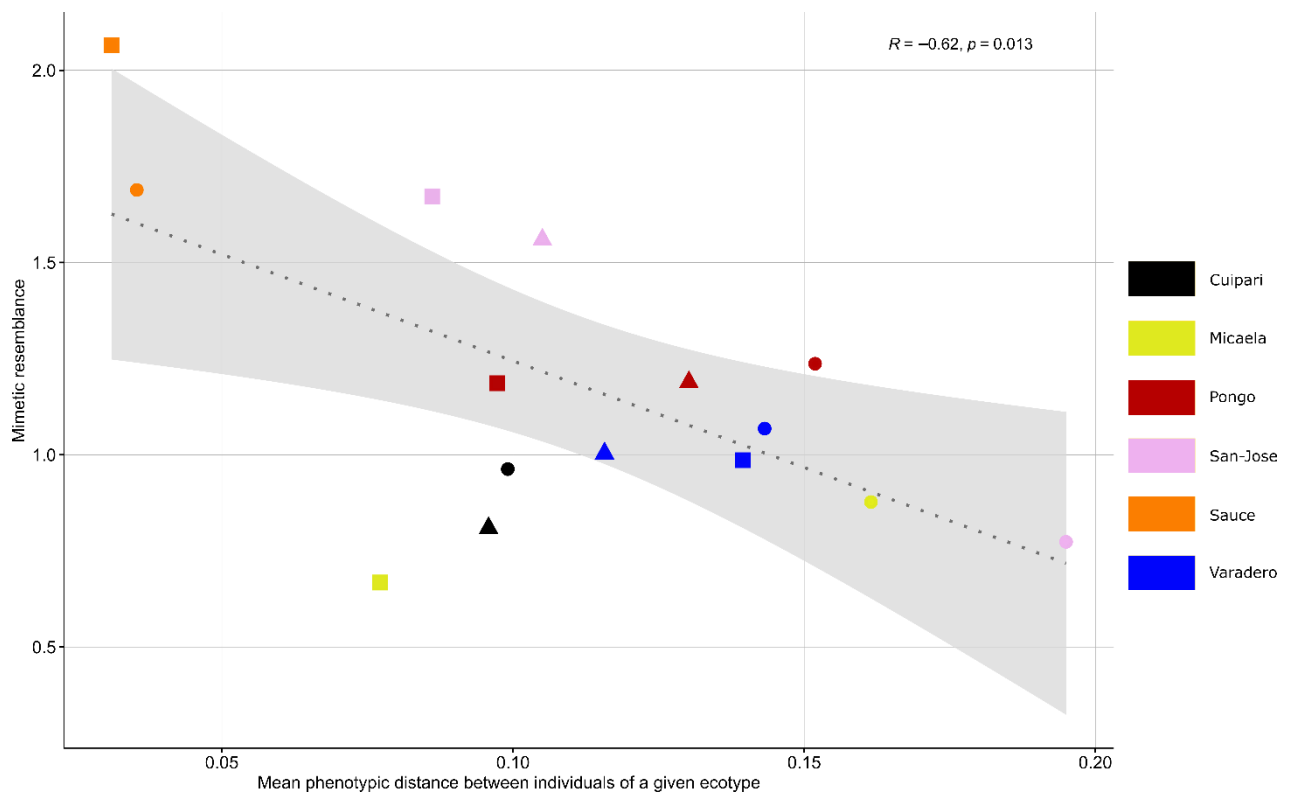
266 All the analyses were performed in R studio (45) using the packages Patternize (42) and Rstatix (46),
267 and plot with GGplot2 (47).

268 *Results*

269 *Relationship between warning signal variability and the degree of mimicry convergence*

270 We tested the relationship between local warning signal variation within species and the
271 degree of mimetic convergence between species and found a mildly significant negative relationship
272 ($P = 0.01308$, $d = 13$, $R = -0.62$) (Fig. 2). Mimicry was highly correlated with the amount of ecotypic

273 variability in a number of sites, including Sauce and San José, where strong mimetic resemblance
 274 (from 1.56 to 2.06) coincides with low phenotypic variability (*Var* from 0.03 to 0.1), and Pongo and
 275 Varadero, where ecotypes are more variable (*Var* from 0.98 to 1.23) and little to no mimicry was
 276 observed (*ms* from 0.98 to 1.23). Interestingly, in San José this applies to only 2 of the 3 co-occurring
 277 species, with the third (*R. fantastica*) being non mimetic (*ms* = 0.77) and phenotypically very variable
 278 (*Var* = 0.19). Similarly, in Micaela, *R. fantastica* is very variable (*Var* = 0.16) and non-mimetic with
 279 *R. imitator*, which itself is much less variable (*Var* = 0.07). Finally, although both species in Cuipari
 280 have little phenotypic variation (*Var* = 0.09), the two are not mimetic at all (*ms* < 0.96).



281
 282 **Figure 2: Correlation between the amount of variability within the warning signal of each**
 283 **ecotypes and mimetic distances between each species pair in a given locality.** Mean phenotypic
 284 distances between individuals of the same ecotype increases with increasing phenotypic variability
 285 within that ecotype, and mimicry resemblance increases with increasing resemblance between
 286 sympatric species. Circles: *R. fantastica* clade, squares: *R. imitator*, triangles: *R. variabilis*, dotted line:
 287 regression line, with 95% interval confidence.

288

289

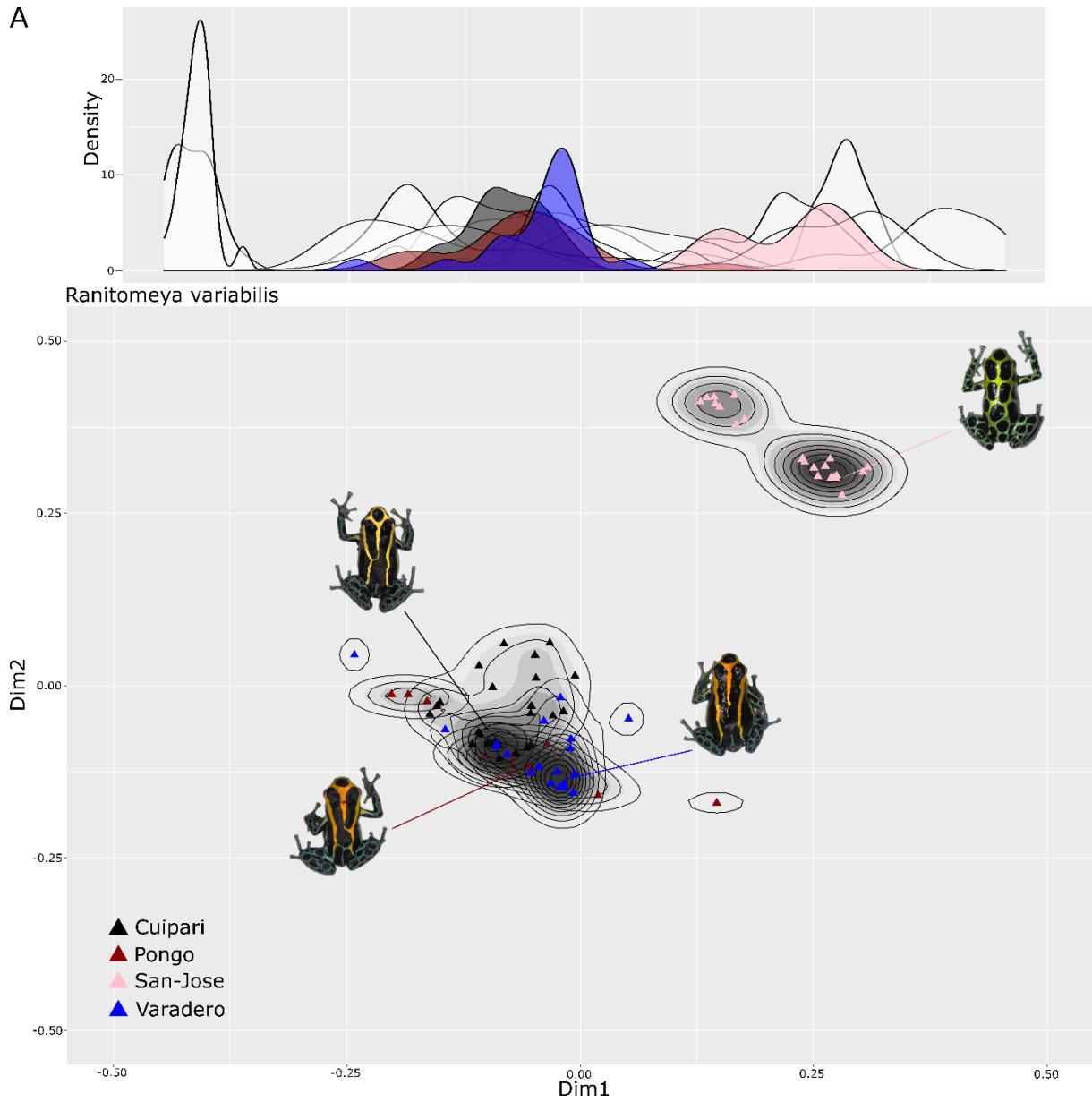
290

291 *Exploring phenotypic variability within and between ecotypes*

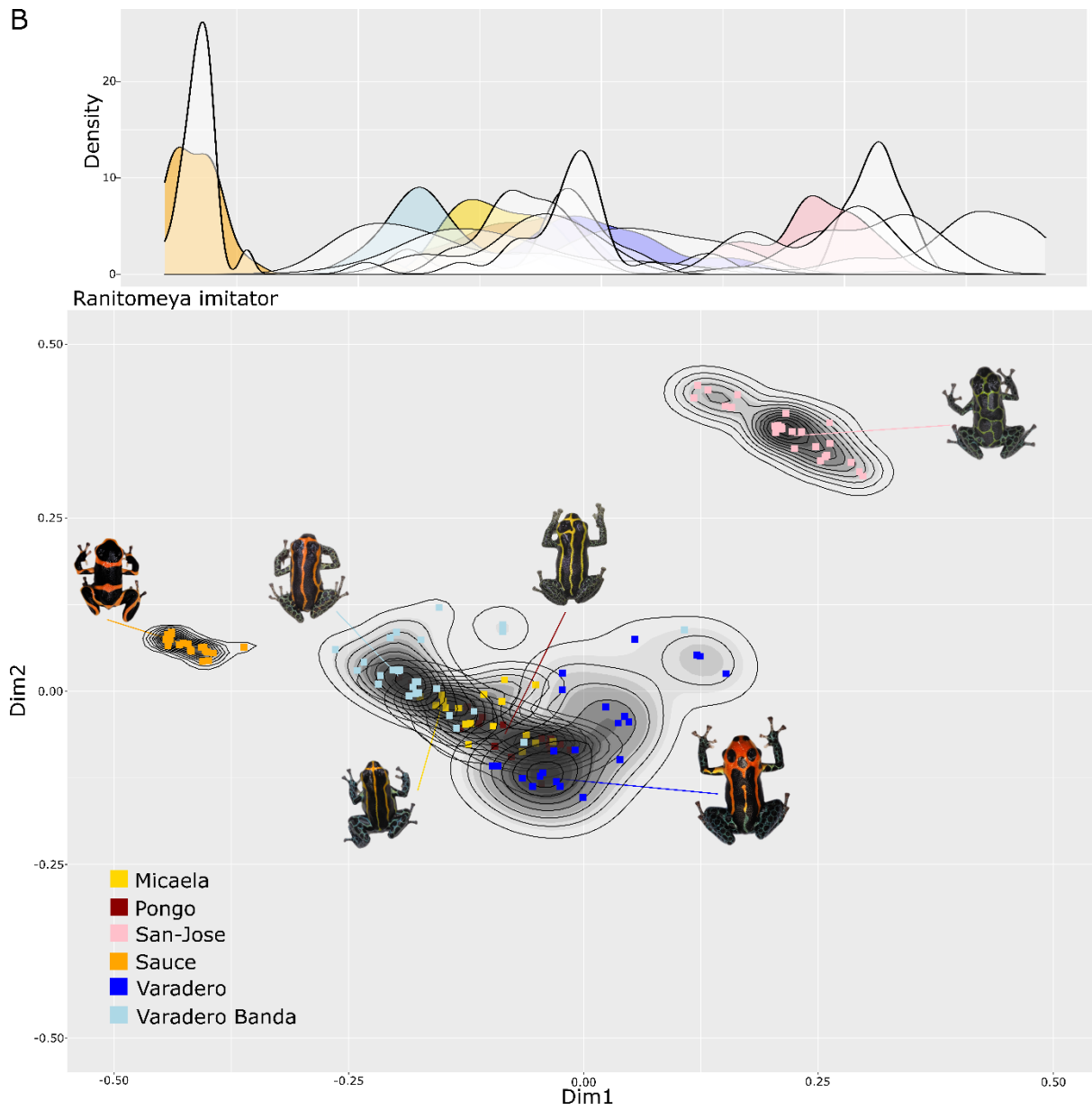
292 We explored the warning signal morphospace generated with the global distance matrix and
293 observed that all three species have 1 or 2 well-differentiated peaks (Fig. 3). These peaks are in the
294 same localities, for the population of Sauce and San José (respectively *R. variabilis* and *R. imitator*),
295 which is consistent with the mimicry relationship observed in the previous section. A binomial
296 distribution of the phenotype is observed in San José, both for *R. variabilis* and *R. imitator*. This
297 mostly results from a reticulation gradient, between highly reticulated to less reticulated, observed in
298 both species. Ecotypes observed in a third site, Cuipari, also display less phenotypic variability (well-
299 defined peaks), but phenotypic resemblance nevertheless overlaps with those of other ecotypes. In
300 fact, with the exception of Sauce and San José, all ecotypes have gradual and overlapping clines, with
301 some individuals resembling nearby peaks rather than their own, and/or appear to be intermediate. For
302 *R. variabilis*, each ecotype overlaps to some degree with the others (Fig. 3. A), whereas for *R. imitator*
303 and *R. fantastica*, phenotypes overlap in a more continuous fashion (Fig. 3. B, C). This is especially
304 true for *R. fantastica*: despite clear peaks for each ecotype, there is also continuous variation along the
305 valleys between peaks. Some individuals (approximately 6% to 29% depending on populations)
306 harbor warning signals that are phenotypically more similar to that of other localities (alternative peak)
307 than they are to the most abundant phenotype in their locality. In some cases, these “outlier”
308 individuals are even found within another locality’s adaptive peak.

309

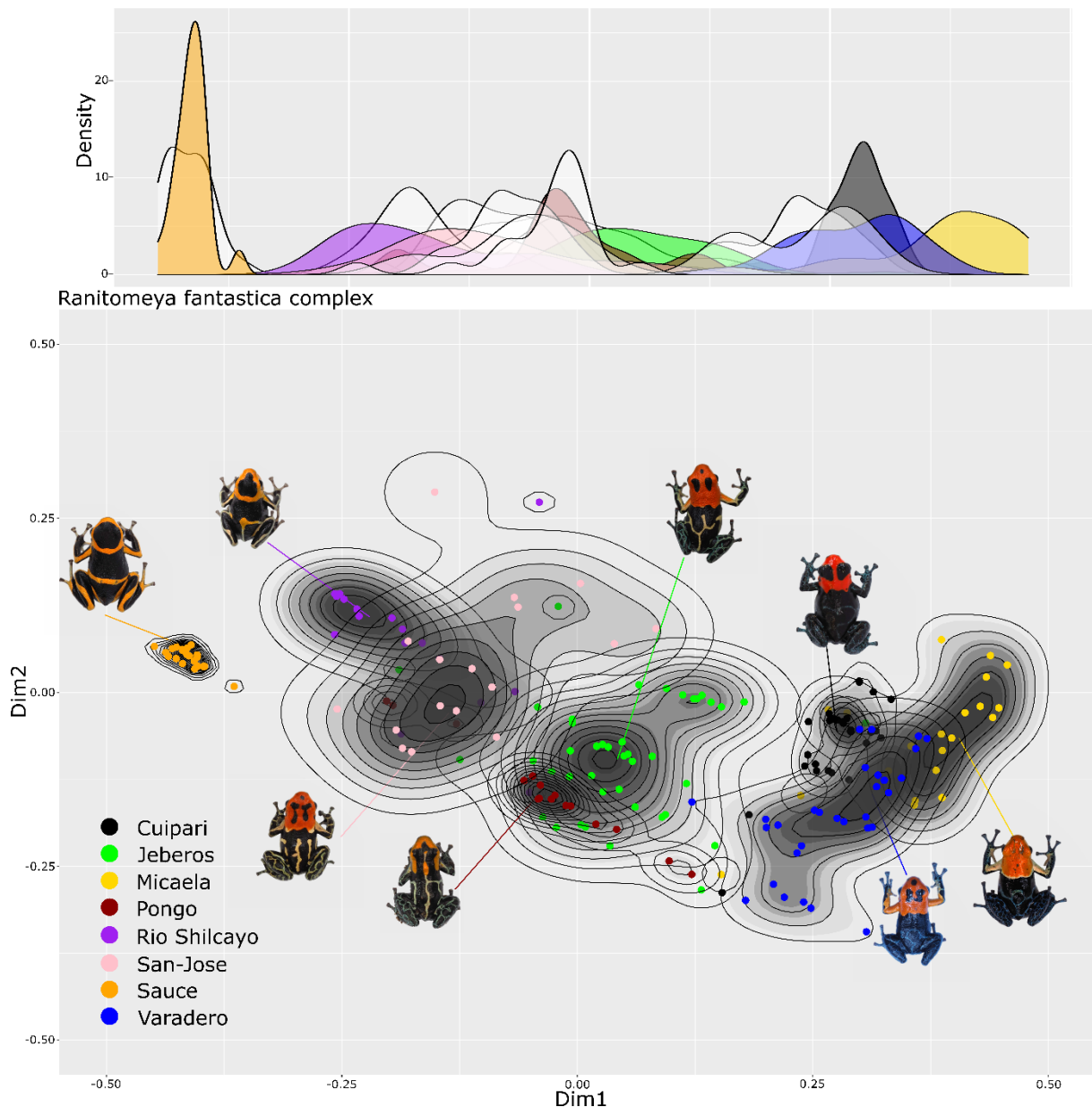
A



310



311



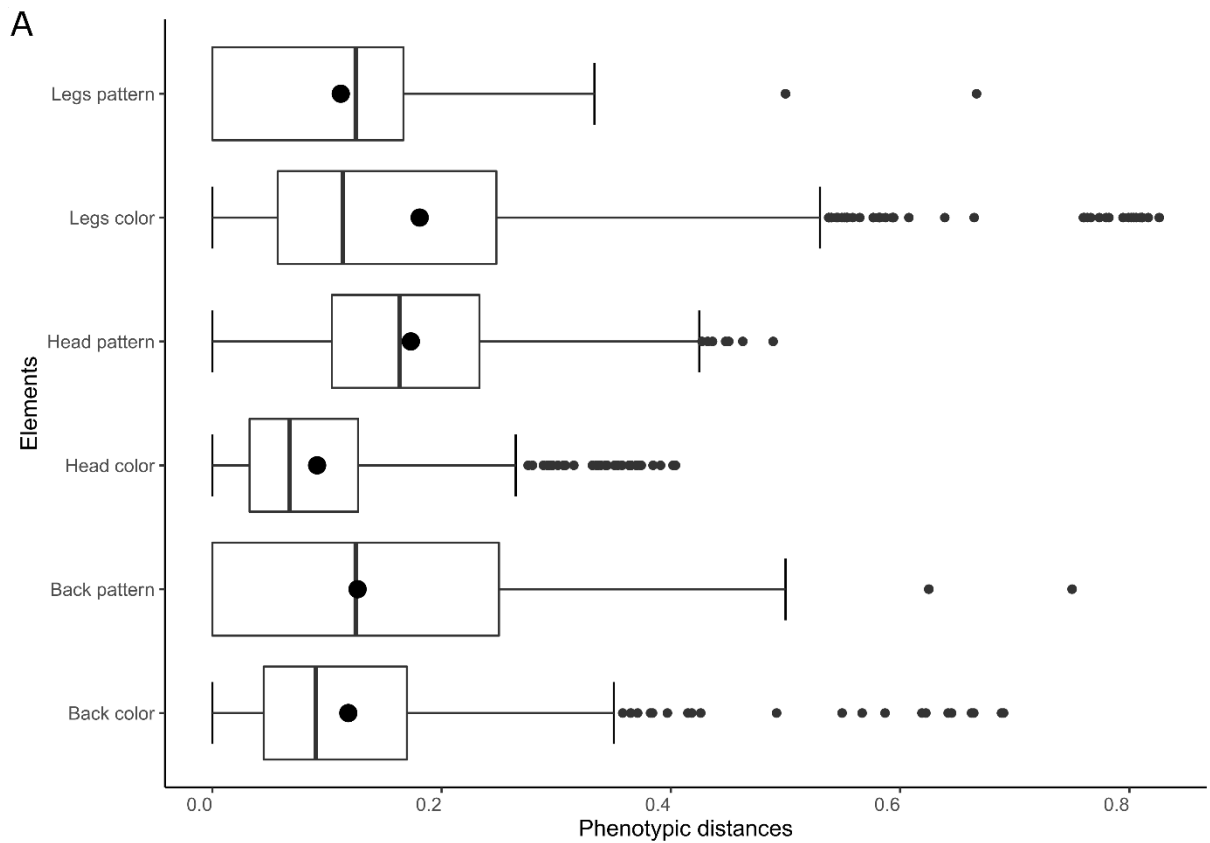
312
 313 **Figure 3: Phenotypic landscape of the warning signal using a frequency plot for A) *R. variabilis***
 314 **(N=84), B) *R. imitator* (N=132) and C) *R. fantastica* clade (N=196), with frequency distribution of**
 315 **all ecotypic phenotypes above. Frequency is shown in colour for the ecotypes of the species shown.**
 316 **Ellipses are polygon bins of the frequency, divided into 10 levels, each of decreasing population**
 317 **frequency. Circle: *R. fantastica* clade, square: *R. imitator*, triangle: *R. variabilis*.**

318

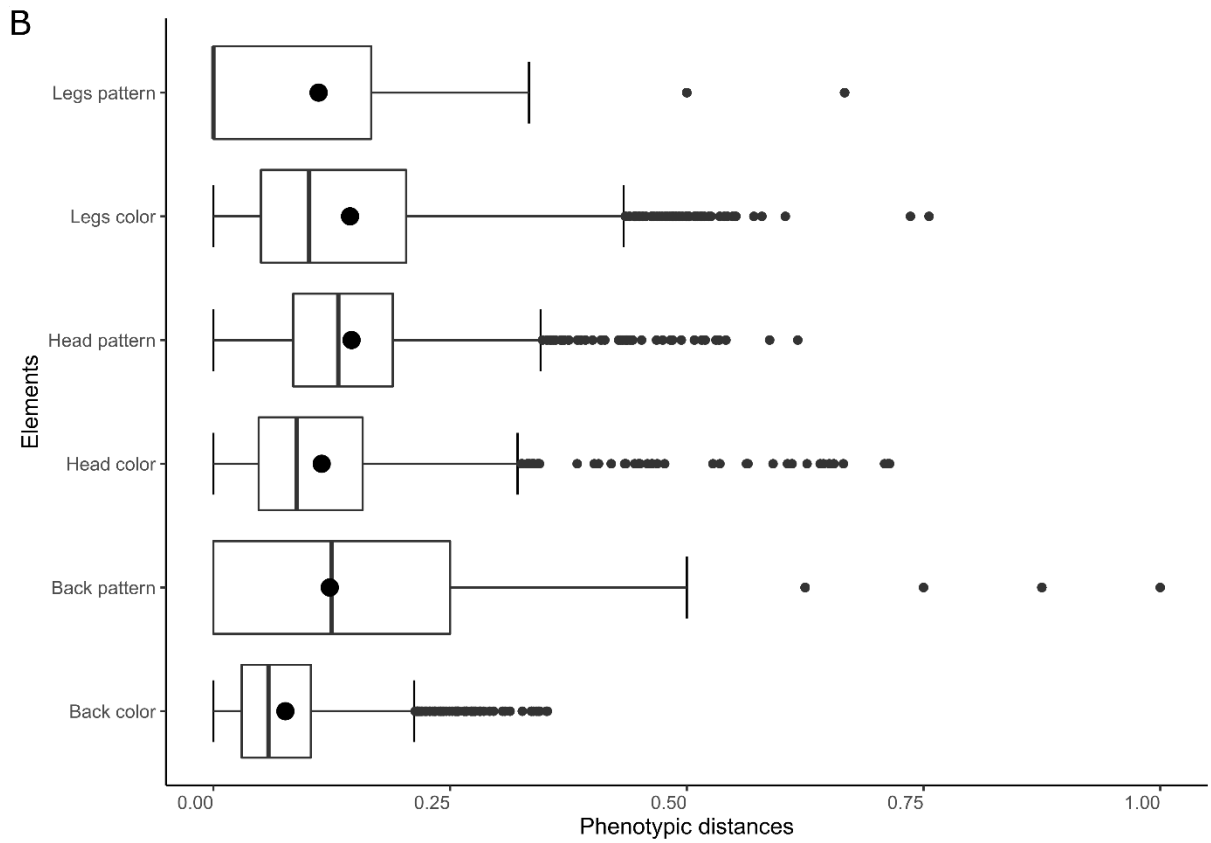
319 ***Variation within colour and/or pattern elements vs whole phenotypes***

320 Of the 6 quantitative phenotypic characteristics measured (head, dorsal and limb colour and
 321 pattern investigated separately), all appears to display variability (variation of characteristics for a
 322 population above 12.6% of the total variation within the study system) for all ecotypes and all species
 323 ($H \geq 115.96$, $df = 5$, $P < 0.005$), as would be expected if some characteristics of the warning signal

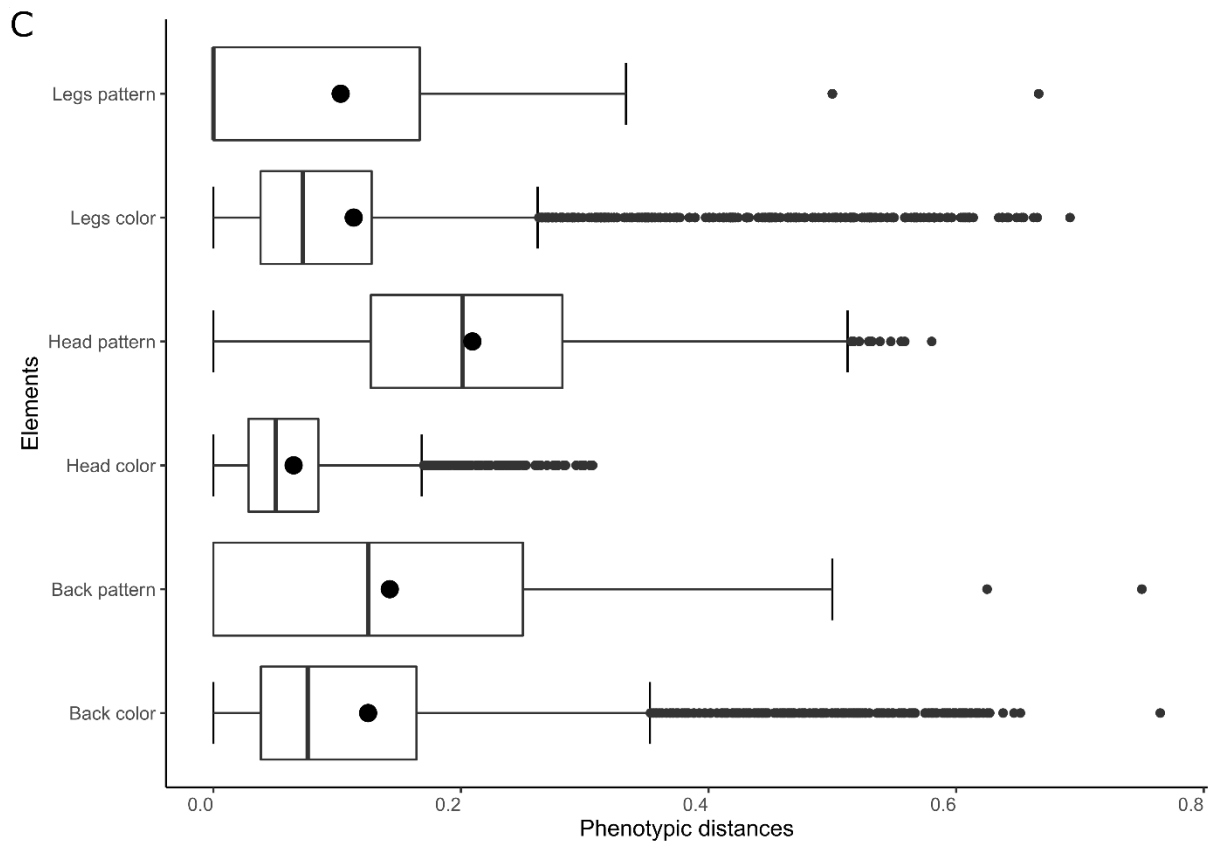
324 were universally under selection (Fig. 4). Even for those localities previously found to be less variable
 325 and more mimetic (i.e. Sauce and San José; Fig. 2), the least variable characteristics of the colour
 326 pattern differed somewhat between species. Nevertheless, overall the back and head colour were
 327 repeatedly found to be the least variable (*mean Var* of all ecotypes < 0.114), and therefore possibly
 328 under the strongest selection, for all ecotypes and all species. This was especially true for ecotypes
 329 with the least variation in the overall warning signal (from Sauce, San José, Cuipari). Furthermore, for
 330 all species and populations, the head pattern was found to be consistently the most variable (*mean Var*
 331 of all ecotypes = 0.188 $P < 0.05$).



332



333



334

335 **Figure 4: Boxplot showing the sum of the variation measured as pairwise phenotypic distance in**
 336 **the warning signal characteristics between every individuals of A) *R. variabilis*, B) *R. imitator***
 337 **and C) the *R. fantastica* clade.**

338

339

340

Discussion

341

342

343

344

345

346

347

348

349

The diversification of adaptive traits strongly depends on the amount of standing variation that is present (1). Aposematism, whereby unprofitable species advertise this fact to predators, often via bright colour patterns, is an extreme case where positive frequency-dependent selection is expected to limit the phenotypic variation present (3), even among species (i.e. mimicry). Yet, numerous aposematic species display extensive geographic divergence in warning signals throughout their range, resulting in a complex and stable spatial mosaic of colour-patterns (13,28,48). Here, by confronting the theoretical expectation of selection on warning signals to the reality of a natural system, we show both the relationship between trait variation and mimicry, and how standing phenotypic variation may be present and facilitate warning signal diversification.

350

351

352

353

354

355

356

357

358

359

360

361

362

The Peruvian *Ranitomeya* system, with its extensive level of geographic diversity and mimicry relationships, enabled us to empirically demonstrate that locally an increase in mimicry resemblance between species is associated with a reduction of phenotypic variation within populations. This negative relationship is consistent with the expectation that both aposematism and Mullerian mimicry are shaped by the same positive frequency-dependent selection in nature (15). Indeed, some localities are presumably categorized by intense selection were characterized by perfect mimicry and low intra-specific variation in warning signal, whereas mimicry was absent or imperfect and warning signals variable within species in other sites, suggesting a relaxation of predator selection and the existence of heterogeneity in the intensity of selection between localities (4). However, notable exceptions to the negative relationship between warning signal variations and mimicry convergence were also observed, namely 1) the coexistence of two species with very distinct and fixed (i.e. non-variable) warning signals, and 2) localities where there is mimetic convergence despite high intraspecific phenotypic variations in their warning signals. Different non-exclusive explanations may exist for this.

363

364

365

366

367

368

369

370

371

First, developmental constraints that differ between species could hinder phenotypic convergence despite intense selection by predators. In *Ranitomeya*, an important number of patterning and pigment genes, principally related to melanophores, melanin, iridophores and guanine synthesis, are responsible for the diverse warning signals. Notable interspecific differences in the gene pathways underlying the production of the same warning signal have recently been demonstrated (49). These differences in the pathways underlying colour pattern development between species is also supported by the recent work of Twomey et al. (2020), which demonstrated that the colour of these frogs results from the physical interaction of nanostructure and pigments, either synthesized or acquired via dietary uptake depending on the species. The production of a novel colour or pattern with a mimetic

372 advantage might therefore be unlikely when the difference between sympatric species is too large
373 (5,50,51).

374 Moreover, the predator community may be able to memorize several warning distinct signals,
375 enabling the coexistence of different aposematic phenotype in sympatry. The coexistence of multiple
376 warning signals has been extensively documented in neotropical butterflies. In these taxa, multiple
377 mimicry rings (*i.e.* mimetic relationship between multiple unrelated species) often coexist in sympatry,
378 with up to a dozen species in at least five mimicry rings for Heliconiinae (52,53). In the mimetic and
379 aposematic Ithomiini butterfly tribe, up to 8 mimicry rings can exist within a given locality (54). The
380 persistence of these mimicry rings could be due to diverse spatio-temporal differences in distribution
381 such as vertical stratification (55,56), distinct nocturnal roosting heights or habits (52), temporal
382 separation in flight activity (57), etc. The different mimicry rings in these butterflies display very
383 different signals and any intermediate forms are selected against (58,59). In *Ranitomeya*, selection
384 against intermediate forms is consistent with the existence of co-occurring but non-mimetic species,
385 where each warning signal is visually very different (See Cuipari for example, Fig. 1 et Fig. 2).

386 On the other hand, large within population signal diversity despite mimicry could result from
387 the migration of new warning signal alleles from other populations. This connectivity between
388 ecotypes has been proposed to maintain phenotypic variation even when the signal is under positive
389 frequency-dependent selection (60,61) (Fig. 2). The effect of gene flow has been documented in a
390 number of study systems, including in the hybrid zones of *Heliconius* (62,63), where large numbers of
391 recombinant phenotypes are observed within long but narrow regions of contact between races.
392 Similar situations have been documented in poison dart frogs, such as in *Oophaga anchicayensis* and
393 *Oophaga lehmannii* (64) and between the mimetic ecotypes of *R. imitator* (35), where hybrid zones are
394 characterized by large amounts of warning signal diversity.

395 Furthermore, predator generalization at the community level could lead to species displaying
396 accurate mimicry despite variability in the warning signal. Generalization can be observed when
397 predators learn to avoid an unpalatable phenotype and as a result will also avoid other more or less
398 similar forms, thus enabling some level of variation to be maintained (33). Indeed, a limited number of
399 key aspects of the warning signal may be used for recognition by predators. While these traits are
400 expected to be less variable, the rest of the colour-pattern might be under relaxed selection and could
401 therefore be more variable. Indeed, our dissection of the warning signal revealed that the head colour,
402 and to a lesser extent the back colour, were the least variable traits, not only within populations
403 characterized by a more variable warning signal (e.g. Cuipari, Pongo and Varadero), but for all
404 populations and species in general (Fig. 4). Patterning, however, was consistently the most variable
405 aspect of the signal.

406 These differences inherent to different components of the warning signal are consistent with
407 other analytical and experimental studies: long-wavelength colours (*i.e.* red, orange and yellow) that
408 characterize the head and dorsum of *Ranitomeya* are thought to provide the most efficient and
409 contrasting signal in the forest environment, making colouration a key aspect of an effective warning
410 signal and under strong stabilizing selection (65). Moreover, bird predators have been shown to
411 preferentially avoid colour rather than other phenotypic elements in aposematic prey (32) enabling
412 those other characteristics to be more variable. Finally, the patterns created by the alternating vivid
413 colour and black background, while surely improving detection and learning by predators, are also
414 easily generalized by avian predators (66). As such, different patterns, such as varying degrees of
415 reticulations, are likely not discriminated by predators, enabling for variations in the local warning
416 signal. Moreover, these patterning elements are likely generated by reaction diffusion mechanisms
417 which result in unique patterns for each individual (43) and even continued change during an
418 individual's lifetime (67). The low diversity of colours compared to patterning elements could also be
419 explained by sexual selection acting to stabilize this trait. While little is known about mate choice in
420 *Ranitomeya*, a major role of colour in communication has been documented in the strawberry poison
421 frog (*Oophaga pumilio*); in this species, females prefer brighter male dorsal colouration with
422 apparently no preference for other warning signal characteristics (68,69).

423 Finally, our analyses of the morphospaces revealed that the phenotypic distribution of each
424 ecotype is not as discrete or as constrained as initially expected (Fig. 3). While a number of discrete
425 mimetic ecotypes are observed (e.g. the green reticulated *R. variabilis* and *R. imitator*, the orange
426 banded *R. imitator* and *R. summersi*), variations within some localities overlap with those of other
427 localities, such that differentiation between ecotypes is blurred. This is especially apparent in the *R.*
428 *fantastica* clade where numerous localities have a few individuals that are distant from the local
429 warning signal optimum (*i.e.* the most abundant one). While the source of this within-locality
430 phenotypic variation cannot currently be ascertained (*i.e.* migration, relaxation of predation, warning
431 signal generalization, etc.), the standing variation present locally may fuel the diversification of
432 warning signals. Population with warning signal variation can be the source of the colonization of a
433 new habitat with a naive predator community and therefore generate the birth of a new ecotype (24).

434 In conclusion, assessing how variations in the warning signals of *Ranitomeya* frogs are
435 structured at the scale of individuals, populations, geography and communities between unrelated
436 species has enabled to challenge and confirm, to some extent, theoretical expectations of natural
437 selection and diversification on this iconic trait. As expected, we show a negative relationship between
438 signal variability and mimetic convergence, demonstrating experimentally that both phenomena are
439 the result of positive frequency selection. But contrary to general expectations, we show that warning
440 signals are much more variable than expected, and that variability between population seems
441 continuous, suggesting the maintaining of this variability among the metapopulation. This standing

442 genetic variation is most likely the key to warning signal diversification: founding effects might
443 strongly contribute to colour pattern diversification.

444

445

446 **References**

- 447 1. Nosil P, Comeault AA, Farkas TE. Ecological Speciation and Its Consequences. In: Encyclopedia
448 of Evolutionary Biology [Internet]. Elsevier; 2016 [cited 2020 Dec 16]. p. 487–93. Available
449 from: <https://linkinghub.elsevier.com/retrieve/pii/B9780128000496000676>
- 450 2. Stroud JT, Losos JB. Ecological Opportunity and Adaptive Radiation. *Annu Rev Ecol Evol Syst.*
451 2016 Nov;47(1):507–32.
- 452 3. Caro T, Ruxton G. Aposematism: Unpacking the Defences. *Trends Ecol Evol.* 2019
453 Jul;34(7):595–604.
- 454 4. Ruxton GD, Sherratt TN, Speed MP. *Avoiding attack: the evolutionary ecology of crypsis,*
455 *warning signals, and mimicry.* Oxford: Oxford Univ. Press; 2004. 249 p.
- 456 5. Sherratt TN. The evolution of Müllerian mimicry. *Naturwissenschaften.* 2008 Aug;95(8):681–95.
- 457 6. Bates HW. XXXII. Contributions to an Insect Fauna of the Amazon Valley. *Lepidoptera:*
458 *Heliconidae.* *Trans Linn Soc Lond.* 1862 Nov;23(3):495–566.
- 459 7. Briolat ES, Burdfield-Steel ER, Paul SC, Rönkä KH, Seymoure BM, Stankowich T, et al.
460 Diversity in warning coloration: selective paradox or the norm?: Diversity in warning coloration.
461 *Biol Rev.* 2019 Apr;94(2):388–414.
- 462 8. Brown KS, Sheppard PM, Turner RG. Quaternary refugia in tropical America: evidence from race
463 formation in *Heliconius* butterflies. *Proc R Soc Lond B Biol Sci.* 1974 Nov 5;187(1088):369–78.
- 464 9. Sheppard PM, Turner JRG, Brown KS, Benson WW, Singer MC. Genetics and the evolution of
465 muellerian mimicry in *heliconius* butterflies. *Philos Trans R Soc Lond B Biol Sci.* 1985 Feb
466 28;308(1137):433–610.
- 467 10. Wang IJ, Shaffer HB. RAPID COLOR EVOLUTION IN AN APOSEMATIC SPECIES: A
468 PHYLOGENETIC ANALYSIS OF COLOR VARIATION IN THE STRIKINGLY
469 POLYMORPHIC STRAWBERRY POISON-DART FROG. *Evolution.* 2008 Nov;62(11):2742–
470 59.
- 471 11. Marek PE, Bond JE. A Mullerian mimicry ring in Appalachian millipedes. *Proc Natl Acad Sci.*
472 2009 Jun 16;106(24):9755–60.
- 473 12. Winters AE, Green NF, Wilson NG, How MJ, Garson MJ, Marshall NJ, et al. Stabilizing selection
474 on individual pattern elements of aposematic signals. *Proc R Soc B Biol Sci.* 2017 Aug
475 30;284(1861):20170926.
- 476 13. Sherratt TN. Spatial mosaic formation through frequency-dependent selection in Müllerian
477 mimicry complexes. *J Theor Biol.* 2006 May;240(2):165–74.

- 478 14. Aubier TG, Sherratt TN. Diversity in Müllerian mimicry: The optimal predator sampling strategy
479 explains both local and regional polymorphism in prey: DIVERSITY IN MÜLLERIAN
480 MIMICRY. *Evolution*. 2015 Nov;69(11):2831–45.
- 481 15. Chouteau M, Arias M, Joron M. Warning signals are under positive frequency-dependent
482 selection in nature. *Proc Natl Acad Sci*. 2016 Feb 23;113(8):2164–9.
- 483 16. Chouteau M, Angers B. The Role of Predators in Maintaining the Geographic Organization of
484 Aposematic Signals. *Am Nat*. 2011 Dec;178(6):810–7.
- 485 17. Holmes IA, Grundler MR, Davis Rabosky AR. Predator Perspective Drives Geographic Variation
486 in Frequency-Dependent Polymorphism. *Am Nat*. 2017 Oct;190(4):E78–93.
- 487 18. Noonan BP, Comeault AA. The role of predator selection on polymorphic aposematic poison
488 frogs. *Biol Lett*. 2009;5.
- 489 19. Hegna RH, Nokelainen O, Hegna JR, Mappes J. To quiver or to shiver: increased melanization
490 benefits thermoregulation, but reduces warning signal efficacy in the wood tiger moth. *Proc R Soc
491 B Biol Sci*. 2013 Mar 22;280(1755):20122812.
- 492 20. Merrill RM, Chia A, Nadeau NJ. Divergent warning patterns contribute to assortative mating
493 between incipient *Heliconius* species. *Ecol Evol*. 2014 Apr;4(7):911–7.
- 494 21. Motyka M, Bocek M, Kusy D, Bocak L. Interactions in multi-pattern Müllerian communities
495 support origins of new patterns, false structures, imperfect resemblance and mimetic sexual
496 dimorphism. *Sci Rep*. 2020 Dec;10(1):11193.
- 497 22. Symula R, Schulte R, Summers K. Molecular phylogenetic evidence for a mimetic radiation in
498 Peruvian poison frogs supports a Müllerian mimicry hypothesis. *Proc R Soc Lond B Biol Sci*.
499 2001 Dec 7;268(1484):2415–21.
- 500 23. Twomey E, Yeager J, Brown J, Morales V, Cummings M, Summers K. Phenotypic and Genetic
501 Divergence among Poison Frog Populations in a Mimetic Radiation. *PloS One*. 2013 Feb
502 6;8:e55443.
- 503 24. Chouteau M, Angers B. Wright's Shifting Balance Theory and the Diversification of Aposematic
504 Signals. Ortiz-Barrientos D, editor. *PLoS ONE*. 2012 Mar 28;7(3):e34028.
- 505 25. Mallet J. Shift happens! Shifting balance and the evolution of diversity in warning colour and
506 mimicry. *Ecol Entomol*. 2010 Jan;35:90–104.
- 507 26. Wright S. The Shifting Balance Theory and Macroevolution. *Annu Rev Genet*. 1982 Dec;16(1):1–
508 20.
- 509 27. Comeault AA, Noonan BP. Spatial variation in the fitness of divergent aposematic phenotypes of
510 the poison frog, *Dendrobates tinctorius*: Spatial variation in fitness. *J Evol Biol*. 2011
511 Jun;24(6):1374–9.
- 512 28. Rönkä K, Valkonen JK, Nokelainen O, Rojas B, Gordon S, Burdfield-Steel E, et al. Geographic
513 mosaic of selection by avian predators on hindwing warning colour in a polymorphic aposematic
514 moth. 2020;11.
- 515 29. Jay P, Whibley A, Frézal L, Rodríguez de Cara MÁ, Nowell RW, Mallet J, et al. Supergene
516 Evolution Triggered by the Introgression of a Chromosomal Inversion. *Curr Biol*. 2018
517 Jun;28(11):1839-1845.e3.

- 518 30. Medina I, Wang IJ, Salazar C, Amézquita A. Hybridization promotes color polymorphism in the
519 aposematic harlequin poison frog, *Oophaga histrionica*. *Ecol Evol*. 2013 Nov;3(13):4388–400.
- 520 31. Pardo-Diaz C, Salazar C, Baxter SW, Merot C, Figueiredo-Ready W, Joron M, et al. Adaptive
521 Introgression across Species Boundaries in Heliconius Butterflies. R. Kronforst M, editor. *PLoS*
522 *Genet*. 2012 Jun 21;8(6):e1002752.
- 523 32. Rönkä K, De Pasqual C, Mappes J, Gordon S, Rojas B. Colour alone matters: no predator
524 generalization among morphs of an aposematic moth. *Anim Behav*. 2018 Jan;135:153–63.
- 525 33. Arias M, Davey JW, Martin S, Jiggins C, Nadeau N, Joron M, et al. How do predators generalize
526 warning signals in simple and complex prey communities? Insights from a videogame. *Proc R Soc*
527 *B Biol Sci*. 2020 Feb 26;287(1921):20200014.
- 528 34. Brown JL, Twomey E, Amézquita A, De Souza MB, Caldwell JP, Lötters S, et al. A taxonomic
529 revision of the Neotropical poison frog genus *Ranitomeya* (Amphibia: Dendrobatidae). *Zootaxa*.
530 2011 Oct 28;3083(1):1.
- 531 35. Twomey E, Vestergaard JS, Venegas PJ, Summers K. Mimetic Divergence and the Speciation
532 Continuum in the Mimic Poison Frog *Ranitomeya imitator*. *Am Nat*. 2016 Feb;187(2):205–24.
- 533 36. Lötters S, Jungfer KH, Henkel FW, Schmidt W. Poison Frogs: Biology, Species & Captive
534 Husbandry [Internet]. Edition Chimaira. 2007 [cited 2022 Aug 1]. 668 p. Available from:
535 <https://www.nhbs.com/poison-frogs-book>
- 536 37. Robertson JM, Robertson AD. Spatial and temporal patterns of phenotypic variation in a
537 Neotropical frog. *J Biogeogr*. 2007;14.
- 538 38. Santos JC, Cannatella DC. Phenotypic integration emerges from aposematism and scale in poison
539 frogs. *Proc Natl Acad Sci U S A*. 2011 Apr 12;108(15):6175–80.
- 540 39. Scholtyssek C, Osorio DC, Baddeley RJ. Color generalization across hue and saturation in chicks
541 described by a simple (Bayesian) model. *J Vis*. 2016 Aug 17;16(10):8.
- 542 40. Maan ME, Cummings ME. Poison Frog Colors Are Honest Signals of Toxicity, Particularly for
543 Bird Predators. *Am Nat*. 2012 Jan;179(1):E1–14.
- 544 41. Dreher CE, Cummings ME, Pröhl H. An Analysis of Predator Selection to Affect Aposematic
545 Coloration in a Poison Frog Species. *PLoS ONE*. 2015 Jun 25;10(6):e0130571.
- 546 42. Van Belleghem SM, Papa R, Ortiz- Zuazaga H, Hendrickx F, Jiggins CD, Owen McMillan W, et
547 al. patternize: An R package for quantifying colour pattern variation. Vamosi J, editor. *Methods*
548 *Ecol Evol*. 2018 Feb;9(2):390–8.
- 549 43. Shigeru K. The reaction-diffusion system: a mechanism for autonomous pattern formation in the
550 animal skin. 2002;7.
- 551 44. Endler JA. A framework for analysing colour pattern geometry: adjacent colours. *Biol J Linn Soc*.
552 2012;107(2):233–53.
- 553 45. R Core Team. R: A language and environment for statistical computing. [Internet]. Vienna,
554 Austria: R Foundation for Statistical Computing; 2020 [cited 2022 Nov 8]. Available from:
555 <https://www.R-project.org/>
- 556 46. Kassambara A. rstatix: Pipe-Friendly Framework for Basic Statistical Tests [Internet]. 2023.
557 Available from: <https://rpkgs.datanovia.com/rstatix/>

- 558 47. Wickham H. ggplot2: Elegant Graphics for Data Analysis [Internet]. Springer-Verlag New York;
559 2016. Available from: <https://ggplot2.tidyverse.org>
- 560 48. Mochida K. A parallel geographical mosaic of morphological and behavioural aposematic traits of
561 the newt, *Cynops pyrrhogaster* (Urodela: Salamandridae): PARALLEL VARIATION IN
562 APOSEMATIC TRAITS. *Biol J Linn Soc.* 2009 Jun 23;97(3):613–22.
- 563 49. Stuckert AMM, Chouteau M, McClure M, LaPolice TM, Linderoth T, Nielsen R, et al. The
564 genomics of mimicry: Gene expression throughout development provides insights into convergent
565 and divergent phenotypes in a Müllerian mimicry system. *Mol Ecol.* 2021 Aug;30(16):4039–61.
- 566 50. Losos JB. CONVERGENCE, ADAPTATION, AND CONSTRAINT. *Evolution.* 2011
567 Jul;65(7):1827–40.
- 568 51. Pigliucci M. Phenotypic integration: studying the ecology and evolution of complex phenotypes.
569 *Ecol Lett.* 2003 Mar;6(3):265–72.
- 570 52. Mallet J, Gilbert L. Why are there so many mimicry rings? Correlations between habitat,
571 behaviour and mimicry in *Heliconius* butterflies. 1995;22.
- 572 53. Papageorgis C. Mimicry in Neotropical butterflies. *Am Sci* [Internet]. 1975 [cited 2022 Jul 21];
573 Available from:
574 https://scholar.google.com/scholar_lookup?title=Mimicry+in+Neotropical+butterflies&author=Pa
575 [pageorgis%2C+C.&publication_year=1975](https://scholar.google.com/scholar_lookup?title=Mimicry+in+Neotropical+butterflies&author=Pa)
- 576 54. Beccaloni G. Ecology, natural history and behaviour of ithomiine butterflies and their mimics in
577 Ecuador (Lepidoptera: Nymphalidae: Ithomiinae). *Trop Lepidoptera.* 1997 Jan 1;8:103–24.
- 578 55. Beccaloni GW. Vertical stratification of ithomiine butterfly (Nymphalidae: Ithomiinae) mimicry
579 complexes: the relationship between adult flight height and larval host-plant height. *Biol J Linn*
580 *Soc.* 1996 Nov 14;62:313–41.
- 581 56. Willmott KR, Robinson Willmott JC, Elias M, Jiggins CD. Maintaining mimicry diversity:
582 optimal warning colour patterns differ among microhabitats in Amazonian clearwing butterflies.
583 *Proc R Soc B Biol Sci.* 2017 May 31;284(1855):20170744.
- 584 57. DeVRIES PJ, Lande R, Murray D. Associations of co-mimetic ithomiine butterflies on small
585 spatial and temporal scales in a neotropical rainforest. *Biol J Linn Soc.* 1999 May;67(1):73–85.
- 586 58. Arias M, le Poul Y, Chouteau M, Boisseau R, Rosser N, Théry M, et al. Crossing fitness valleys:
587 empirical estimation of a fitness landscape associated with polymorphic mimicry. *Proc R Soc B*
588 *Biol Sci.* 2016 Apr 27;283(1829):20160391.
- 589 59. McClure M, Mahrouche L, Houssin C, Monllor M, Le Poul Y, Frérot B, et al. Does divergent
590 selection predict the evolution of mate preference and reproductive isolation in the tropical
591 butterfly genus *Melinaea* (Nymphalidae: Ithomiini)? Eizaguirre C, editor. *J Anim Ecol.* 2019
592 Jun;88(6):940–52.
- 593 60. Mallet J, McMillan WO, Jiggins CD. Mimicry and Warning Color at the Boundary between Races
594 and Species. 1998;15.
- 595 61. Rojas B. Behavioural, ecological, and evolutionary aspects of diversity in frog colour patterns:
596 Function and evolution of frog colour diversity. *Biol Rev.* 2017 May;92(2):1059–80.
- 597 62. Rosser N, Dasmahapatra KK, Mallet J. Stable *Heliconius* butterfly hybrid zones are correlated
598 with a local rainfall peak at the edge of the Amazon basin. *Evolution.* 2014;68(12):3470–84.

- 599 63. Van Belleghem SM, Cole JM, Montejo-Kovacevich G, Bacquet CN, McMillan WO, Papa R, et al.
600 Selection and gene flow define polygenic barriers between incipient butterfly species [Internet].
601 Evolutionary Biology; 2020 Apr [cited 2022 Jul 13]. Available from:
602 <http://biorxiv.org/lookup/doi/10.1101/2020.04.09.034470>
- 603 64. Ebersbach J, Posso- Terranova A, Bogdanowicz S, Gómez- Díaz M, García- González MaX,
604 Bolívar- García W, et al. Complex patterns of differentiation and gene flow underly the
605 divergence of aposematic phenotypes in *Oophaga* poison frogs. Mol Ecol. 2020 Jun;29(11):1944–
606 56.
- 607 65. Arenas LM, Troscianko J, Stevens M. Color contrast and stability as key elements for effective
608 warning signals. Front Ecol Evol [Internet]. 2014 [cited 2022 Jul 18];2. Available from:
609 <https://www.frontiersin.org/articles/10.3389/fevo.2014.00025>
- 610 66. Aronsson M, Gamberale-Stille G. Evidence of signaling benefits to contrasting internal color
611 boundaries in warning coloration. Behav Ecol. 2013 Mar 1;24(2):349–54.
- 612 67. Yuan ML, Jung C, Bell RC, Nelson JL. Aposematic patterns shift continuously throughout the life
613 of poison frogs. J Zool. 2022;1–8.
- 614 68. Maan ME, Cummings ME. Sexual dimorphism and directional sexual selection on aposematic
615 signals in a poison frog [Internet]. 2009 [cited 2022 Jul 18]. Available from:
616 <https://www.pnas.org/doi/10.1073/pnas.0903327106>
- 617 69. Maan ME, Cummings ME. Female preferences for aposematic signal components in a
618 polymorphic poison frog. Evol Int J Org Evol. 2008 Sep;62(9):2334–45.

619

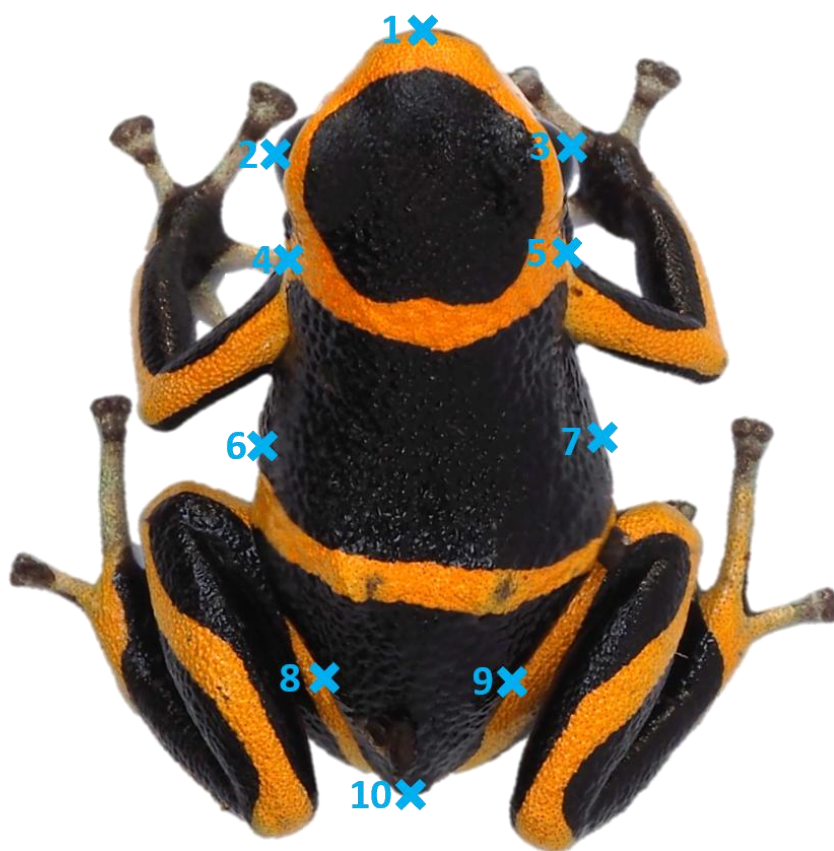
620 Supplementary

621 **Supplementary Table 1: localities name and their coordinate for each ecotype and number of**
622 **individuals sampled.**

			Species				
					<i>fantastica</i> clade		
			<i>R. variabilis</i>	<i>R. imitator</i>	<i>R. fantastica</i>	<i>R. summersi</i>	<i>R. benedicta</i>
Locality	Coord						
	X	Y					
Cuipari	-5,96275565	-75,982599	25	.	.	.	29
Jeberos	-5,673449999	-76,13710545	.	.	41	.	.
Michaela	-5,967441177	-76,13710545	.	18	21	.	.
Pongo	-6,327966642	-76,2843848	17	17	16	.	.
Rio Shilcayo	-6,481749946	-76,36888627	.	.	.	17	.
San-Jose	-6,404399192	-76,37855216	22	25	17	.	.
Sauce	-6,691179088	-76,23698863	.	25	.	23	
Varadero	-5,732839762	-76,44980548	20	22	30	.	.
Varadero Banda	-6,031769819	-76,67983393	.	26	.	.	.

623

624



625

626 **Supplementary figure 1: Landmark position used to align the frogs pictures.**

627