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Advergence in Müllerian mimicry: the case of the poison dart frogs of Northern Peru revisited

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Whether the evolution of similar aposematic signals in different unpalatable species (i.e. Müllerian mimicry) is because of phenotypic convergence or advergence continues to puzzle scientists. The poison dart frog *Ranitomeya imitator* provides a rare example in support of the hypothesis of advergence: this species was believed to mimic numerous distinct model species because of high phenotypic variability and low genetic divergence among populations. In this study, we test the evidence in support of advergence using a population genetic framework in two localities where *R. imitator* is sympatric with different model species, *Ranitomeya ventrimaculata* and *Ranitomeya variabilis*. Genetic analyses revealed incomplete sorting of mitochondrial haplotypes between the two model species. These two species are also less genetically differentiated than *R. imitator* populations on the basis of both mitochondrial and nuclear DNA comparisons. The genetic similarity between the model species suggests that they have either diverged more recently than *R. imitator* populations or that they are still connected by gene flow and were misidentified as different species. An analysis of phenotypic variability indicates that the model species are as variable as *R. imitator*. These results do not support the hypothesis of advergence by *R. imitator*. Although we cannot rule out phenotypic advergence in the evolution of Müllerian mimicry, this study reopens the discussion regarding the direction of the evolution of mimicry in the *R. imitator* system.

Keywords: phenotypic advergence; *Ranitomeya*; mimicry

1. INTRODUCTION

The diversification of aposematic signals in Müllerian mimicry is a puzzling phenomenon, because mimicry is expected to promote uniformity in warning signals [1]. Yet substantial variation in aposematic signals has been shown in, for example, Neotropical butterflies [2] and poison dart frogs [3] throughout their range. Such diversification raises the question of whether both sympatric species change towards a common

phenotype (i.e. convergence) or if the phenotype of one species moves towards the phenotype of another species that remains constant (i.e. advergence) as expected in Batesian mimicry [4]. Advergence is considered more likely when one species' aposematic signal is established before the arrival of a second species [3,5]. Empirical evidence, principally available indirectly for the putative case of *Ranitomeya imitator*, has thus far tended to support advergence [6,7].

Symula *et al.* [3] concluded, using a small number of individuals per population, that the phenotypically variable *R. imitator* mimics distinct model species in different geographical areas. This was supported by the small divergence measured between mitochondrial haplotypes of different *R. imitator* colour morphs compared with that observed between model species, suggesting that *R. imitator* had undergone a relatively recent geographical radiation during which it adopted the aposematic signals of the distinct model species it encountered [3].

However, owing to the importance of this textbook example in the field of evolutionary biology of mimicry [6,7], we re-addressed the theory of advergence in the *R. imitator* system using population genetics, in the localities originally used for the description of the mimicry association [3]. We compared two adjacent but phenotypically distinct populations of *R. imitator* and its local sympatric model species, *Ranitomeya ventrimaculata* and *Ranitomeya variabilis*. We specifically test the following predictions in support of advergence: (i) the model species display higher genetic differentiation when compared with *R. imitator* populations, and (ii) *R. imitator* is more phenotypically variable within and between regions than the model species [5].

2. MATERIAL AND METHODS

The sampling was performed in the department of San Martín (Peru) in two adjacent localities separated by *ca* 16 km (figure 1). A minimum of 15 individuals per species were caught in these localities (table 1). Individuals were digitally photographed under standard conditions next to a black–grey–white card for colour standardization, and a toe clipping was collected for genetic analyses.

The control region was used to assess the genetic diversity of the mitochondrial genome. The primers 5'-AATGTATATGCCAT-TATC-3' and 5'-GAAATATTATAGACCTATATC-3' designed on *Oophaga pumilio* sequences [8] successfully amplified a segment of 274 bp for *R. imitator* and 273 bp for both model species (GenBank accession nos. JF340126–JF340141). Haplotype frequencies were assessed using single strand conformation polymorphism and the distinct haplotypes were sequenced. Sequence alignment was performed using CLUSTALW. Genetic differentiation between populations was evaluated with both haplotype frequency (F_{ST}) and sequences (ϕ_{ST}) using ARLEQUIN v. 3.1 [9]. ARLEQUIN was also used to infer a haplotype minimum spanning network and to assess the mean number of mutations between populations.

Genetic diversity of the nuclear genome was assessed with nine highly variable microsatellite loci: RimiA06, RimiA07, RimiB01, RimiB02b, RimiB07b, RimiB11, RimiC05b, RimiE02b, RimiF06 for *Ranitomeya imitator* and four loci: RvarA09, RvarB01, RvarF08 and RvarG12 for both *R. variabilis* and *R. ventrimaculata* according to Brown *et al.* [10]. Exact tests for Hardy–Weinberg equilibrium, differentiation in allele frequencies and estimation of F_{ST} and R_{ST} were computed using ARLEQUIN v. 3.1 [9]. The relevance of these metrics (nuclear DNA (nDNA) F_{ST} versus R_{ST} and mitochondrial DNA (mtDNA) F_{ST} versus ϕ_{ST}) was tested using SPAGED1 [11]. STRUCTURE v. 2.3 [12] was used without prior population information to confirm population organization. Runs were performed with a burn-in length and Markov chain Monte Carlo of 100 000 generations and 10 runs were performed for $K = 2–5$. The best number of clusters K was *a posteriori* determined according to Evano *et al.* [13].

The individual photographs were corrected for ambient light colour and the dominant hue of the Hue, Saturation and Brightness colour domain was used to quantify the conspicuous dorsal coloration of individuals [14]. Each individual was then assigned to one of the three

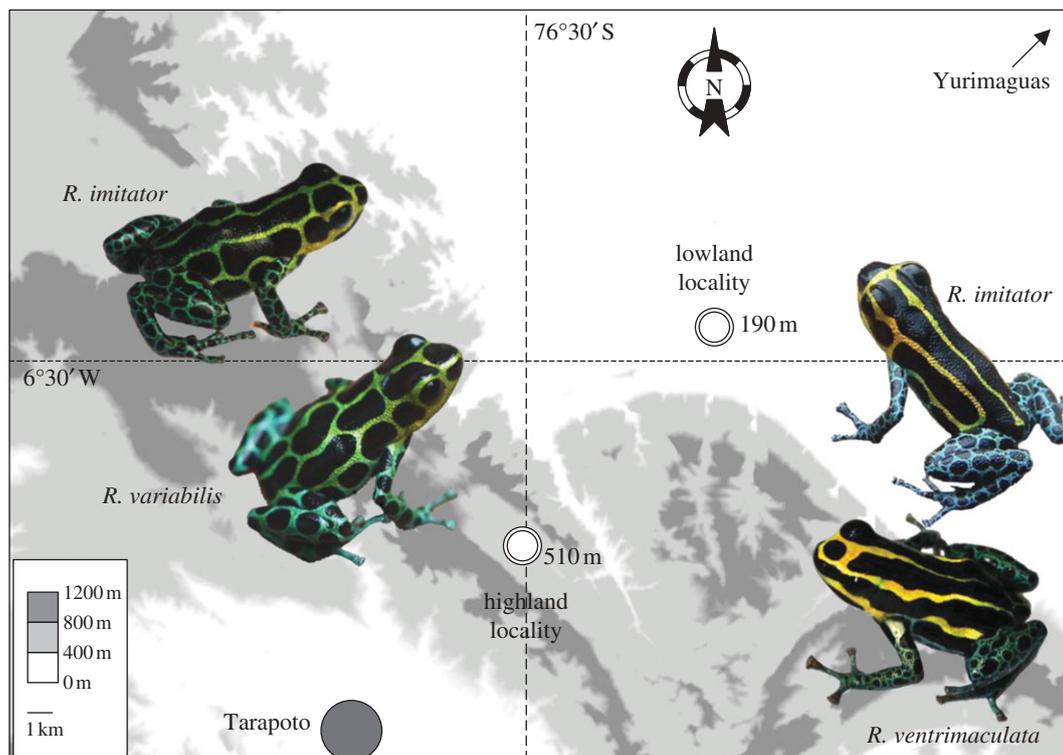


Figure 1. Map of the study area showing the localities under investigation and the phenotypes of the paired *Ranitomeya* species.

Table 1. Genetic variability and differentiation estimated from nuclear and mitochondrial markers between populations of *Ranitomeya imitator* and the model species. (The associated probabilities after 999 random permutations are indicated in parenthesis. Test for relevance of molecular information is included for both the nuclear loci and mitochondrial control region.)

	<i>R. imitator</i>		model species	
	lowland <i>n</i> = 20	highland <i>n</i> = 22	<i>R. ventrimaculata</i> (lowland) <i>n</i> = 15	<i>R. variabilis</i> (highland) <i>n</i> = 16
nuclear				
mean number of alleles per loci (mean \pm s.d.)	11.2 \pm 2.1	11 \pm 3.4	12.5 \pm 3.3	11 \pm 2.83
H_E (mean \pm s.d.)	0.863 \pm 0.065	0.861 \pm 0.038	0.879 \pm 0.044	0.803 \pm 0.123
F_{ST} (95% CI)	0.103 (0.092–0.118) ($p < 0.001$)		0.023 (0–0.041) ($p = 0.034$)	
	0.074 (0.051–0.098) ($p < 0.001$) ^a			
R_{ST} (95% CI)	0.206 (0.123–0.266) ($p < 0.001$)		0.039 (0–0.072) ($p = 0.200$)	
	0.128 (0.098–0.157) ($p < 0.001$) ^a			
probability that $R_{ST} > F_{ST}$	$p = 0.002$		$p = 0.523$	
mitochondrial				
number of haplotypes	7	3	3	4
H_D (mean \pm s.d.)	0.901 \pm 0.076	0.432 \pm 0.132	0.670 \pm 0.081	0.591 \pm 0.121
F_{ST}	0.343 ($p < 0.001$)		0.083 ($p = 0.069$)	
φ_{ST}	0.596 ($p < 0.001$)		0.115 ($p = 0.019$)	
probability that $\varphi_{ST} > F_{ST}$	$p = 0.042$		$p = 0.438$	

^aDifferentiation measured using the four loci displaying the lowest differentiation.

following qualitative bins based on their hue: light orange (30°–60°), yellow (61°–90°) and green (91°–120°). The dorsal pattern in the localities studied consisted of three well-separated longitudinal stripes with different degrees of reticulation between them. Each individual was assigned to a qualitative bin based on the amount of reticulation: no reticulation (0), slightly reticulated (1–2), reticulated (3–4) and highly reticulated (5+). Comparison of variance between sites was assessed using Levene's test (W).

3. RESULTS

Both the mitochondrial and nuclear markers displayed high diversity within populations (table 1). The

mitochondrial minimum spanning network (figure 2a) revealed that populations of *R. imitator* are characterized by distinct haplotypes, with a mean number of 1.84 mutations between populations. On the other hand, the haplotypes of *R. ventrimaculata/variabilis* are intermingled and both species had one common haplotype in high frequency, resulting in a number of mutations (0.14) one order of magnitude lower than that of *R. imitator*. All populations were in Hardy–Weinberg equilibrium for nuclear markers and exact tests of differentiation are significant ($p < 0.001$). The estimates of

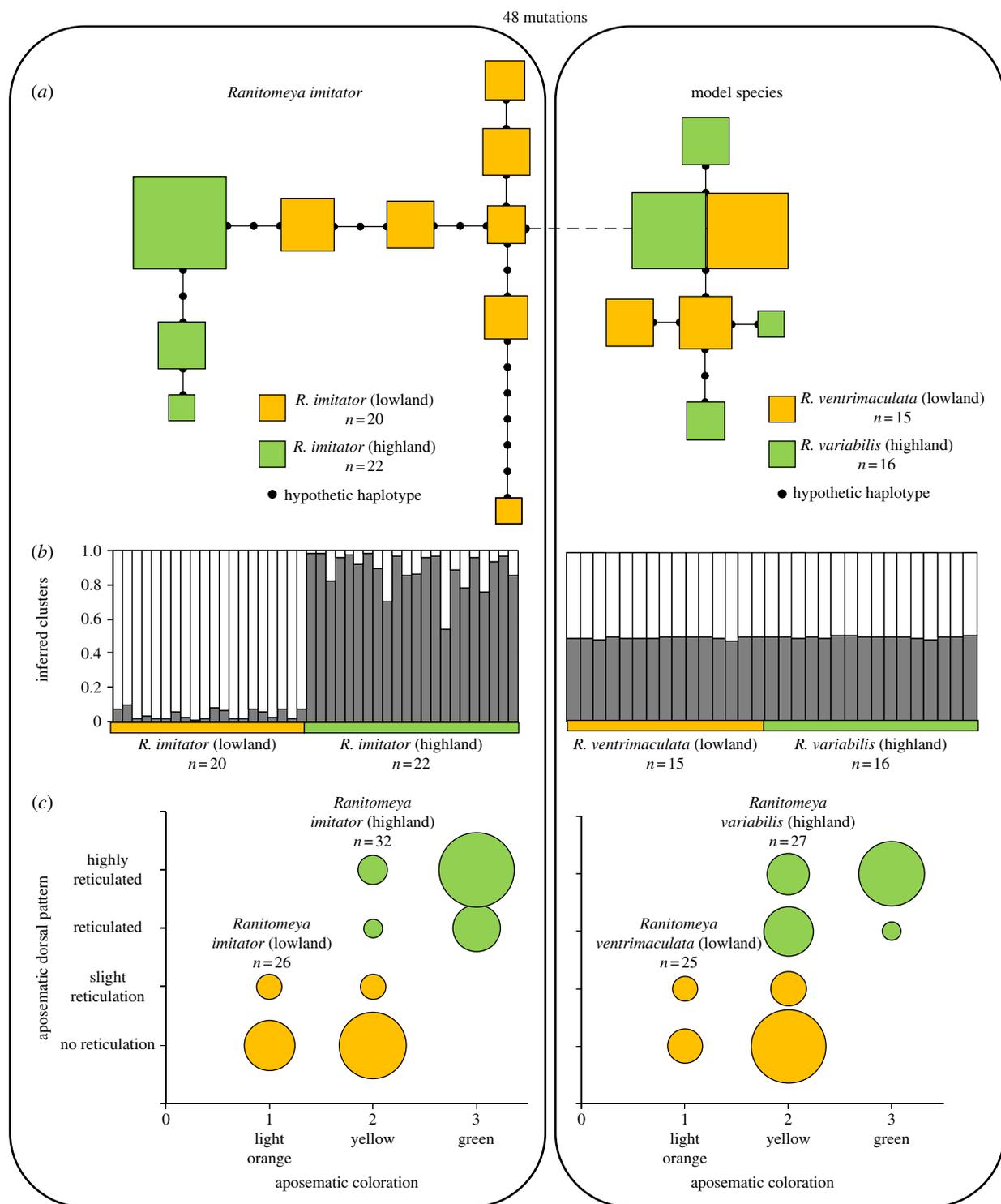


Figure 2. Genetic and phenotypic differences between the *Ranitomeya imitator* populations and between model species. (a) Minimum spanning network inferred from the mitochondrial DNA haplotypes. Each box represents a distinct haplotype and its size refers to the relative abundance within populations. (b) Individual assignment among $K = 2$ clusters (black and white) using STRUCTURE and (c) frequency distribution of the different aposomatic phenotypes (as colours and dorsal patterns) found within the different populations. The size of the circles represents the relative abundance of the phenotypes.

differentiation assessed with mtDNA and nDNA (table 1) are higher between populations of *R. imitator* than between the model species, even when the four loci displaying the lowest differentiation are used to compensate for the different number of loci available between species. SPAGEDI results confirm that the use of the mutational information (F_{ST} versus R_{ST}) is relevant for *R. imitator* but not between model species (table 1). This is concordant with the STRUCTURE results,

where *R. imitator* individuals are clearly separated into two populations according to their geographical location, while individuals of *R. ventrimaculata/variabilis* failed to be properly assigned to their species of origin for all K -values tested (figure 2b).

The comparison of phenotypic variability between *R. ventrimaculata* and *R. imitator* living in the lowland localities ($W_{1,6} = 0.194$, $p = 0.675$) and between *R. variabilis* and *R. imitator* in the highland ($W_{1,6} =$

0.286, $p = 0.612$) show that both species display similar levels of variation and that they share the same aposematic signal in each locality (figure 2c). When the results for both localities are pooled, *R. imitator* and *R. ventrimaculata/variabilis* exhibit the same level of phenotypic variability ($W_{1,14} = 0.007$, $p = 0.936$).

4. DISCUSSION

The predictions of the advergence hypothesis in *R. imitator*, regarding genetic differentiation and phenotypic variability [3,5], were not supported by the results of the present study.

Results of this study revealed that, in spite of a clearly distinct aposematic signal [15], *R. ventrimaculata* and *R. variabilis* display very low genetic differentiation. Incomplete sorting of mtDNA haplotypes, F_{ST} values not significantly different from zero and the inability to assign individuals to its species of origin (STRUCTURE) clearly indicate a recent separation of these groups. While it is not possible in the absence of data on reproductive isolation to determine whether *R. ventrimaculata* and *R. variabilis* are populations of the same species or distinct species, we can conclude that these two groups were recently connected by a common ancestor in both of these scenarios. This is consistent with previous phylogenetic analyses which show that *R. ventrimaculata* is a polyphyletic taxa characterized by the *R. variabilis* and *R. ventrimaculata* from our studied localities being closely related but clearly different from other *R. ventrimaculata* [16].

These findings are important because the identification of the mimicking species is in part based on phenotypic variability; i.e. the mimicking species is expected to be more phenotypically variable across localities than its model species [5]. Since *R. ventrimaculata* and *R. variabilis* belong to the same lineage and have most probably diverged recently, they are in fact as variable as *R. imitator* in the present system.

Another striking result is that the populations of *R. imitator* are far more genetically differentiated than those of *R. ventrimaculata/variabilis* for both genomes. This result is inconsistent with the results of Symula *et al.* [3], whose objective was to assess the Müllerian mimicry relationship but not its direction. The difference is most probably the result of the small number of individuals analysed by Symula *et al.* [3], which prevented them from accurately assessing the levels of differentiation between these genetically diverse species. This casts doubt on the hypothesis that populations of *R. imitator* diversified after the model species and adopted their aposematic signal [3]. To assess the directionality in mimicry, the chronology of founding events in a given locality is essential. However, it is also important to note that genetic differentiation may represent an inappropriate measure of this chronology in the absence of complete phylogeographic information.

Symula *et al.* [3] also discussed a third species, *Ranitomeya summersi* (previously called *Ranitomeya fantastica*), which is mimicked by another colour morph of *R. imitator*. Because this clade is genetically very distinct from both *R. imitator* and *R. ventrimaculata/variabilis*, it could be argued that genetic differences between the model species remain higher than among the *R. imitator* colour morphs.

However, numerous other colour variants in the *R. fantastica* clade have been discovered since the initial description of the system, some of which are sympatric and involved in mimicry with both *R. imitator* and *R. ventrimaculata/variabilis* [17]. Because all species involved in this Müllerian mimicry show high phenotypic variability, conclusions from phenotypic variability or genetic distances between clades remain inconclusive in this system.

In conclusion, our results cast doubt on the evidence previously used to infer the hypothesis of mimetic advergence in the *Ranitomeya* species, as our results contradict two key predictions used in the initial description of the system. This study is of particular importance because the *R. imitator* system is commonly considered to provide the strongest empirical evidence for advergence [6,7]. This does not mean that the theory of advergence is false or that *R. imitator* is not the mimic, but rather that no empirical evidence exists as of yet. This study reopens the discussion concerning the direction of mimicry in the *R. imitator* system.

The experiments were approved by the University of Montreal's ethics committee and by the Instituto Nacional de Recursos Naturales del Peru which provided the collecting and export permits: no. 005-2008-INRENA-IFFS-DCB and CITES no. 11067.

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