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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, 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 species display less 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).

The control region was used to assess the genetic diversity of the mitochondrial genome. The primers 5'-AATGTATATGCCATTATC-3' and 5'-GAAATATTAGACCTATTC-3' designed on *Ophaga pumilo* sequences [8] successfully amplified a segment of c. 1600 bp for both *R. imitator* and *R. variabilis* (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 ($d_{xy}$) using ARLEQUIN v. 3.1 [9]. ARLEQUIN was also used to infer a haplotype 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, 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, distance matrix-based estimation of allele frequencies and $F_{ST}$ and $R_{ST}$ were computed using ARLEQUIN v. 3.1 [9]. The relevance of these metrics (nuclear DNA ($n$DNA) $F_{ST}$ versus $R_{ST}$ and mitochondrial DNA (mtDNA) $F_{ST}$ versus $d_{xy}$) was tested using SPAGEDi [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 Evanno 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
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

![Map of the study area showing the localities under investigation and the phenotypes of the paired *Ranitomeya* species.](image-url)
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 (\( W^2_{1.4} = 0.007, \ p = 0.936 \)).

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 (\( \text{STRUCTURE} \)) 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 \textit{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 \textit{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 \textit{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 \( R. \) fantastics 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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