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Molecular phylogenetic evidence for a mimetic radiation in Peruvian poison frogs supports a Müllerian mimicry hypothesis

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Examples of Müllerian mimicry, in which resemblance between unpalatable species confers mutual benefit, are rare in vertebrates. Strong comparative evidence for mimicry is found when the colour and pattern of a single species closely resemble several different model species simultaneously in different geographical regions. To demonstrate this, it is necessary to provide compelling evidence that the putative mimics do, in fact, form a monophyletic group. We present molecular phylogenetic evidence that the poison frog *Dendrobates imitator* mimics three different poison frogs in different geographical regions in Peru. DNA sequences from four different mitochondrial gene regions in putative members of a single species are analysed using parsimony, maximum-likelihood and neighbour-joining methods. The resulting hypotheses of phylogenetic relationships demonstrate that the different populations of *D. imitator* form a monophyletic group. To our knowledge, these results provide the first evidence for a Müllerian mimetic radiation in amphibians in which a single species mimics different sympatric species in different geographical regions.

Keywords: Müllerian mimic; poison frog; *Dendrobates imitator*

1. INTRODUCTION

The evolution of mimicry represents a microcosm of evolutionary biology, with ramifications in fields as diverse as population genetics, speciation, sexual selection, biodiversity and community ecology (Joron & Mallet 1998). Müller's (1879) hypothesis that toxic species would obtain a selective advantage by sharing similar colours and patterns has generated great interest and considerable controversy among scientists (Mallet & Joron 1999; Speed & Turner 1999). Most of the evidence for Müllerian mimicry comes from studies of insects (e.g. Brower 1994; Kapan 2001).

Examples of Müllerian mimicry are rare in vertebrates, although putative examples occur in snakes (Greene & McDiarmid 1981) and salamanders (Brandon *et al*. 1979). In frogs there is little evidence for any form of mimicry, although a few cases have been suggested as possible examples (Batesian mimicry: Lamar & Wild (1995); Müllerian mimicry: Silverstone (1976)).

The absence of evidence for mimicry in frogs is puzzling because many species of frog are toxic (Pough 1988). Poison frogs of the family Dendrobatidae are generally extremely poisonous, producing some of the most toxic alkaloid poisons known (Daly *et al*. 1987). Experiments using mice indicate that minute quantities of many of the dendrobatid toxins are lethal to vertebrates if they enter the bloodstream (Daly & Myers 1967; Myers *et al*. 1978). Experiments involving both vertebrate and invertebrate predators have demonstrated the unpalatability of these frogs (Daly *et al*. 1978; Szelistowski 1985). These frogs show bright coloration, which is generally believed to be aposematic (Myers & Daly 1983). Recent comparative analysis supports the hypothesis of aposematism (Summers & Clough 2001).

Strong comparative evidence for mimicry is found when different populations within a single species mimic different model species (Pough 1988). For example, the colubrid snake *Erythrolamprus guentheri* is a Batesian mimic of two different species of venomous coral snake (*Micrurus*) in two different geographical regions of Peru (Greene & McDiarmid 1981; Pough 1988).

Schulte (1986) described a poison frog, *Dendrobates imitator*, from the mountains near Tarapoto, Peru, that was remarkably similar to a sympatric species, *Dendrobates variabilis*. These species share virtually identical colour and pattern, with a black background and bright yellow–gold reticulation covering the head, dorsum and flanks, and blue–green reticulation on the legs and venter (figure 1). Only a few differences allow the two species to be distinguished: egg coloration, male advertisement calls and minor differences in coloration, whereby the black spot on the tip of the snout is split by gold reticulation in *D. imitator* but not in *D. variabilis*. A third dendrobatid species, *Dendrobates fantasticus*, with a different colour pattern (an orange–gold chevron on the top of the head, and white markings on the body and legs) also occurs near Tarapoto (Schulte 1999).

Recently, Schulte (2001) described poison frogs from several other populations that have a similar call and egg colour to *D. imitator*, but differ in colour and pattern. Interestingly, each of these populations is sympatric with another species of poison frog that shares similar colour and pattern. One such example involves the Huallaga Canyon, where a population with the same call and egg colour as *D. imitator* co-occurs with a population of *D. fantasticus* (*D. variabilis* is absent from this region). *D. fantasticus* is black with calligraphic yellow–gold

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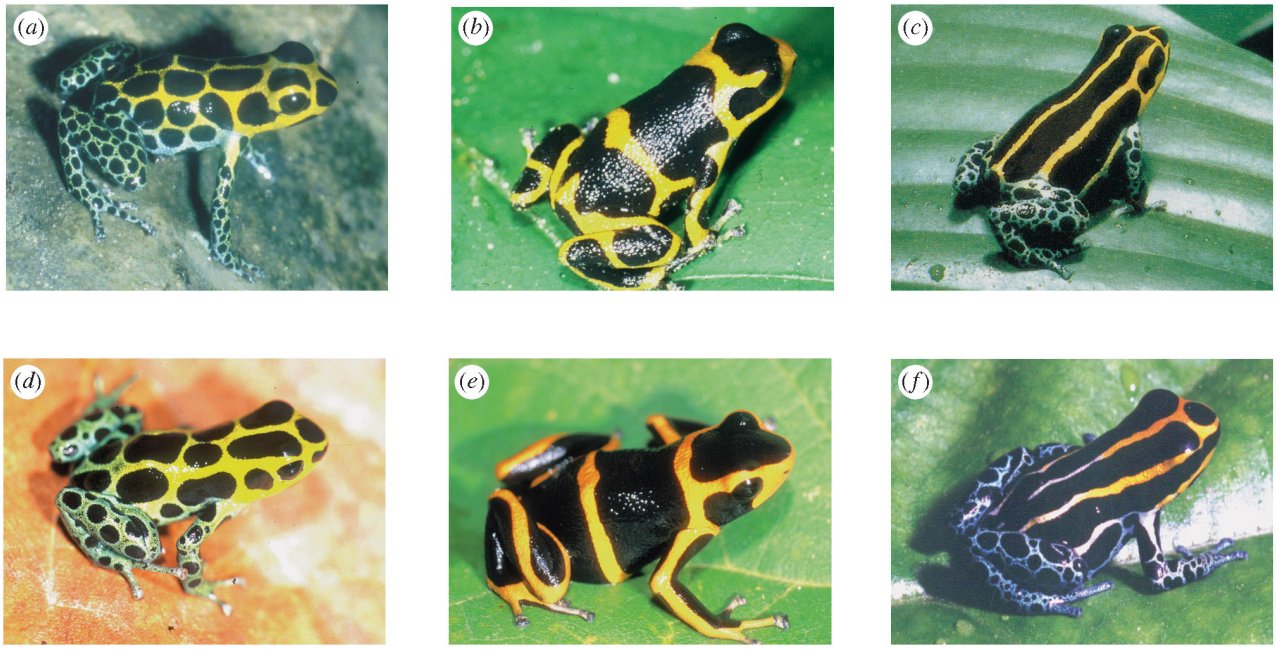

Figure 1. (*a–c*) The three frogs are all putative members of a single species, *Dendrobates imitator*. Each of these different morphs is sympatric with a different species in a different geographical region. The species with which each morph is sympatric is shown directly below that morph. From left to right, the species in (*d–f*) are: *Dendrobates variabilis* (Tarapoto), *Dendrobates fantasticus* (Huallaga Canyon) and '*Dendrobates ventrimaculatus*' (Yurimaguas).

stripes when found in the Huallaga Canyon and on the south side of the Huallaga River, in contrast to their colour and pattern near Tarapoto (figure 1).

Another population of poison frogs putatively assigned to *D. imitator* occurs in the lowlands near Yurimaguas (figure 2). This population has linear gold–yellow stripes on a black dorsum (figure 1). It occurs sympatrically with a population of poison frogs that resemble *Dendrobates ventrimaculatus* (a widespread Amazonian species). Hereafter we will refer to this population as '*D. ventrimaculatus*'. This species occurs only in the lowlands, and is not found near Tarapoto or in the Huallaga Canyon.

If these putative *D. imitator* populations belong to a single species mimicking several different species across different geographical regions, then this would imply a mimetic radiation similar to that of *E. guentheri* and *Micrurus*. However, such a radiation would probably be Müllerian rather than Batesian in nature. Skin extracts from *D. ventrimaculatus*, *D. imitator* and *D. variabilis* all contain potent alkaloid toxins (Daly *et al*. 1987; Schulte 2001). Although *D. fantasticus* has not been analysed for skin toxins, its close relative *Dendrobates reticulatus* is highly toxic (Daly *et al*. 1987).

To demonstrate that a single species is, in fact, mimicking several different species in different regions, it is necessary, first, to provide compelling evidence that the putative mimics form a monophyletic group. To test this hypothesis, we examined DNA sequence variation among members of the 'mimicry pairs' from the three regions involved in this putative mimetic radiation (Tarapoto, Yurimaguas and the Huallaga Canyon), as well as additional populations of *D. imitator* from Chazuta, Shapaja and Sedamillo in the Cordillera Oriental (these populations are similar in pattern to *D. variabilis*) and from lowland areas along the Shanusi River and the lower reaches of the Huallaga Canyon (these populations are similar in pattern to '*D. ventrimaculatus*'). We also sampled several populations of *D. fantasticus*. The hypothesis of mimicry predicts that the populations of *D. imitator*, regardless of colour and pattern, will form a monophyletic clade, and will not be closely related to the sympatric populations that they resemble.

2. METHODS

(a) *Sample collection*

Tissue samples were collected from the following species and populations (sample sizes in parentheses): *D. imitator*: Tarapoto (2), Sedamillo (1), Chazuta (1), Shanusi (1), Lower Huallaga Canyon (2), Huallaga Canyon (3), Shapaja (1), Yurimaguas (2); *D. fantasticus*: Tarapoto (2), between Tarapoto and Shanusi (1), Sauce (3), Huallaga Canyon (2); *D. variabilis*: Tarapoto (2); '*D. ventrimaculatus*': Yurimaguas (2). Samples were taken by removing the tip of a toe from each frog. A few samples were also taken from tadpole tail muscle. The general localities of the populations sampled are shown in figure 2.

(b) *DNA extraction, DNA amplification and sequencing*

Genomic DNA was extracted from tissue samples preserved in preservation buffer (dimethyl sulphoxide–NaCl–ethylene diaminetetra-acetic acid) using the Qiagen DNeasy tissue kit (Qiagen Inc, Valencia, CA, USA). For the 16S ribosomal RNA (rRNA), 12S rRNA, cytochrome *b* and cytochrome oxidase I mitochondrial gene regions, DNA samples were amplified using the DNA primers and protocols described in Summers *et al*. (1999*a*) and Clough & Summers (2000).

Several primers were designed for this study, as follows: DfCOIa (ATTCTTCCGGGTTTTGGTATCATCT), DfCOIb (AAAAAGTTAGGTTGACTCCGGCAAA), DiCOIa (CTTAT TCTTCCTGGTTTCGGATC), DiCOIb (GATTGACACCTAC AAATATKACACCAA), Df12Sa (TGCTCAGTCGTAAACTT

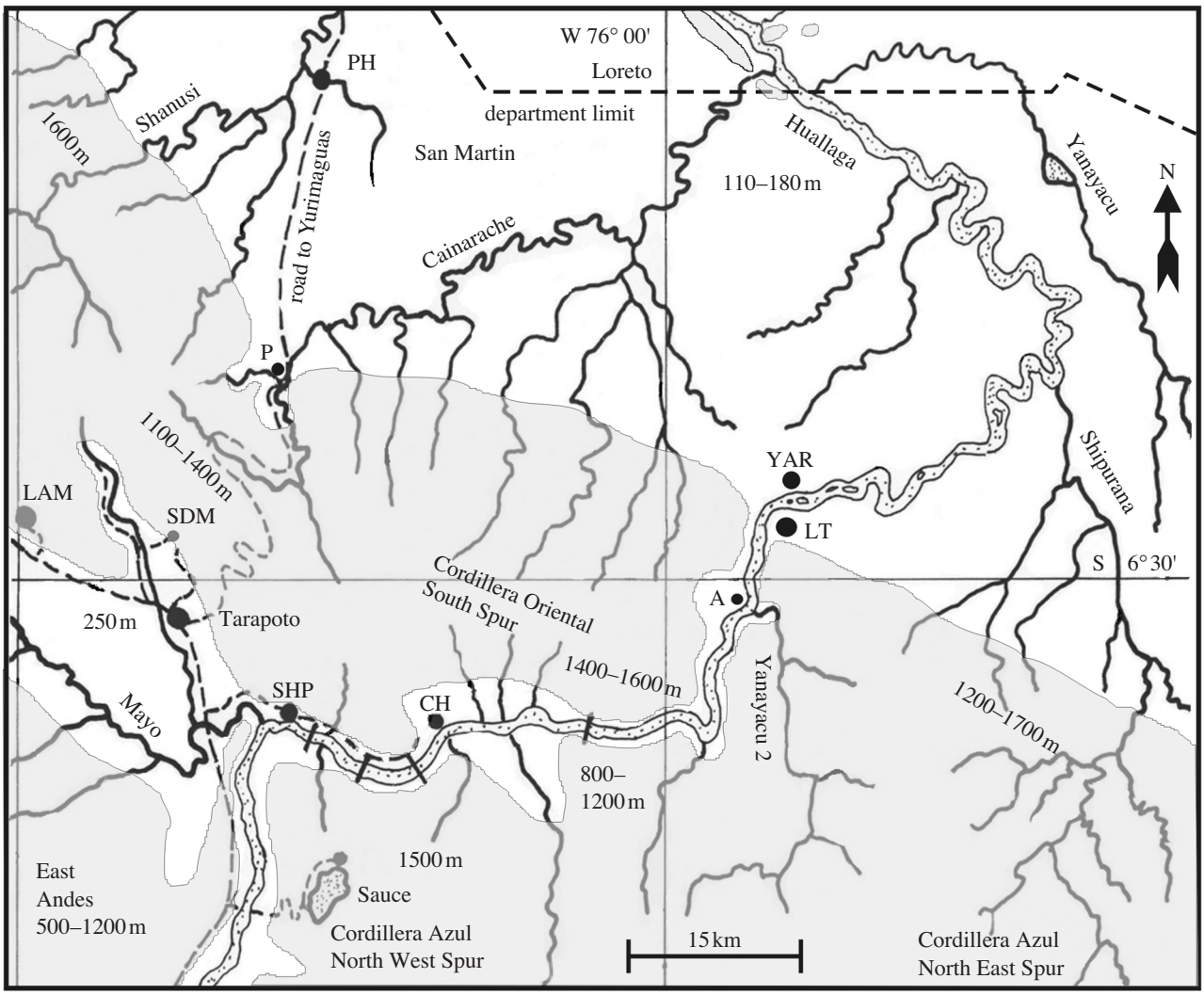

Figure 2. Map of the localities of the dendrobatid-frog tissue samples collected for this research in San Martin, Peru. The scale is *ca*. 1:500 000. Abbreviations are as follows: A, Achinamisa; PH, Allianza, Pampa Hermosa; LAM, Lamas; LT, Leticia; P, La Perla (Pongo de Cainarachi); SHP, Shapaja; CH, Chazuta; YAR, Yarina; SDM, Sedamillo. The Huallaga Canyon region referred to in the text is between Chazuta and Achinamisa.

TAAATTAC) and Df12Sb (AGTGCCGGGTTAAAAAGGACT). The GenBank accession numbers for the DNA sequences used are AF412411–AF412522.

Polymerase chain reaction (PCR) amplifications were purified using the Qiagen's QIAquick PCR purification kit (Qiagen QIAquick PCR purification kit, Qiagen Inc.). Products were sequenced using ABI PRIZM (Perkin-Elmer Corporation, Foster City, CA, USA) sequencing kit. Samples were then prepared for sequencing as in Clough & Summers (2000).

(c) *Sequence analysis*

Each sample was sequenced in both directions, and complementary sequences were aligned using the Applied Biosystems AUTOASSEMBLER v. 1.4.0 (Applied Biosystems 1995). Consensus sequences were transferred to Gene Jockey (Taylor 1990) for alignment with a sequence from the same region in a different individual. Protein-coding sequences were translated to confirm that they were in proper reading frame.

DNA sequences were aligned using Clustal X (Thompson *et al*. 1997). For the cytochrome oxidase I and cytochrome *b* gene regions the alignments were unambiguous with no gaps. For the 16S and 12S rRNA gene regions a few regions of ambiguous alignment were removed from the analysis. Unambiguous informative gaps were coded as transversions in the analysis. One informative gap was found for the 16S gene region.

(d) *Phylogenetic analysis*

Using the program PAUP* v. 4.0 (Swofford 1998), we carried out three kinds of phylogenetic analyses to investigate evolutionary relationships: parsimony, neighbour joining and maximum likelihood.

Parsimony analysis was carried out using stepwise addition with 50 random addition replicates, using tree bisection and reconnection for branch rearrangement. The analysis employed a dynamic weighting scheme to control for variations in the rates of transition and transversion (Williams & Fitch 1990). We did not downweight third base pair substitutions in the analysis because saturation plots of Kimura two-parameter '*p*' distances (Kimura 1980) revealed considerable signal in both third base pair transversions and transitions (Broughton *et al*. 2000).

For the neighbour-joining and parsimony analyses, support for particular nodes was investigated using bootstrapping (Felsenstein 1985) with 2000 replicates. For the outgroups we used *Dendrobates histrionicus* (Ecuador) and *Dendrobates leucomelas* (Venezuela), which fall outside a distinct Amazonian clade within *Dendrobates* (Clough & Summers 2000). We also included two

species of *Dendrobates* from the Amazonian clade: *D. ventrimaculatus* from Ecuador and *D. reticulatus* from Peru.

The neighbour-joining analysis employed the Kimura two-parameter model (Kimura 1980) to calculate genetic distances. Rate variation was modelled using a gamma distribution parameter (Yang 1996) estimated from the data via maximum likelihood.

The congruence of phylogenetic signal among the DNA datasets (cytochrome oxidase I, cytochrome *b*, 16S rRNA and 12S rRNA) was investigated using the partition homogeneity test implemented in PAUP*. We found significant differences between the datasets in terms of the most parsimonious topology favoured by the different gene regions. Parsimony analysis of each gene-region dataset separately revealed that the key differences occurred with respect to the relative placement of three major clades. The cytochrome oxidase I dataset favoured a topology grouping the *D. fantasticus*–*D. reticulatus* clade with the *D. imitator* clade, whereas the 16S and 12S rRNA gene regions both favoured grouping the *D. fantasticus*–*D. reticulatus* clade with the *D. variabilis*–*D. ventrimaculatus* clade. These distinctions are not relevant to the main question addressed in this paper (the monophyly of the *D. imitator* clade, which was supported in all of the separate analyses), so we combined the entire dataset in subsequent analyses.

For the maximum-likelihood analysis, we first determined which model of DNA substitution provided the best fit to our data, using likelihood ratio tests implemented via the program MODELTEST 3.04 (Posada & Crandall 1998). We determined that the best fit to our data was provided by the General Time Reversible (GTR) model of substitution (Rodríguez *et al*. 1990) and a gamma parameter (Yang 1996) of 0.6339 estimated from the data via maximum likelihood. The proportion of invariant sites (0.3393) and the base composition (A = 0.26840, C = 0.24240, G = 0.18970, T = 0.29950) were also estimated via MODELTEST. These parameters were used in a phylogenetic analysis of the data using maximum likelihood in PAUP*. We carried out 10 random-order sequence addition replicates to control for the effects of sequence addition order. Branch swapping was carried out with the tree bisection and reconnection option implemented in PAUP*.

In this study, we have *a priori* phylogenetic hypotheses that are consistent or inconsistent with the mimicry hypothesis. The mimicry hypothesis predicts that each presumptive species (including *D. imitator*) will form a monophyletic group. Hence, the phylogenetic tree consistent with this hypothesis is simply each putative member of each species within a single polytomy, and each species connected to each other species at a basal polytomy. The alternative hypothesis predicts that one or more populations putatively assigned to *D. imitator* are, in fact, more closely related to a different species (*D. fantasticus*, *D. variabilis* or '*D. ventrimaculatus*'), with which they occur sympatrically and which they resemble closely in colour and pattern. This can be specified by simply placing the hypothesized mimics in a monophyletic group with their putative models. In § 3 we test the mimicry hypothesis against each of the alternative hypotheses using Kishino–Hasegawa tests (Kishino & Hasegawa 1989) as implemented in PAUP*.

We also used the Shimodaira–Hasegawa test (Shimodaira & Hasegawa 1999) to investigate differences between *a posteriori* topological hypotheses derived from the analysis of our data (for a discussion of the use of this test, see Goldman *et al*. (2000)). In order to do this, we compared the best tree from the unconstrained maximum-likelihood analysis with maximum-likelihood trees found by three constrained maximum-likelihood analyses: a constraint grouping *D. fantasticus* from the Huallaga Canyon with its putative mimic; a constraint grouping *D. variabilis* from Tarapoto with its putative mimic; and a constraint grouping '*D. ventrimaculatus*' from Yurimaguas with its putative mimic. Hence, we included these four trees in the set of *a posteriori* hypotheses to be compared using the Shimodaira–Hasegawa test.

3. RESULTS

The final dataset for all four gene regions consisted of 1496 base pairs of DNA (487 from cytochrome oxidase I, 282 from cytochrome *b*, 445 from 16S rRNA and 282 from 12S rRNA), out of which 343 were informative. The parsimony analysis of the combined dataset produced 1386 most parsimonious trees. However, the variations between most parsimonious trees involved only rearrangements of the relationships between populations of *D. imitator*. All the investigated populations of *D. imitator* fell within a strongly supported monophyletic clade, with a bootstrap value of 100%. Figure 3 shows the best tree topology and branch-length estimates from the maximum-likelihood analysis. This tree is entirely compatible with the results of the parsimony analysis, and is one of the most parsimonious trees. The best tree from the distance analysis was virtually identical to that from the maximum-likelihood analysis, differing only in a few rearrangements of poorly supported groupings within species.

Populations of *D. fantasticus* formed a strongly supported monophyletic group in all analyses. Members of a population near Sauce were genetically distinct from the other populations. This is consistent with the observation that this population is separated from the others by a major barrier, the Huallaga River. *D. reticulatus* was placed as the sister group to *D. fantasticus* in all analyses. *D. variabilis* from Tarapoto and '*D. ventrimaculatus*' from Yurimaguas formed a monophyletic group, which was closely related to *D. ventrimaculatus* from Amazonian Ecuador.

The *D. fantasticus*–*D. reticulatus* clade was placed with the *D. imitator* clade in all analyses, but with low bootstrap support (< 50%) in the parsimony and distance analyses (the maximum-likelihood analysis was not bootstrapped). This is consistent with the conflict between the datasets (see § 2d).

The Kishino–Hasegawa tests showed highly significant differences between the *a priori* topology predicted by the mimicry hypothesis and each of the *a priori* alternative topologies (table 1). The Shimodaira–Hasegawa test showed highly significant differences between the unconstrained maximum-likelihood tree and each of the constrained alternatives (table 1). The topology consistent with the mimicry hypothesis (grouping all of the populations of *D. imitator* as sister taxa) is significantly better than any alternative topology grouping a population of *D. imitator* with the species that it is mimicking.

4. DISCUSSION

Our molecular phylogenetic analysis supports the hypothesis that different populations of *D. imitator* mimic different species of poison frogs, providing evidence for a

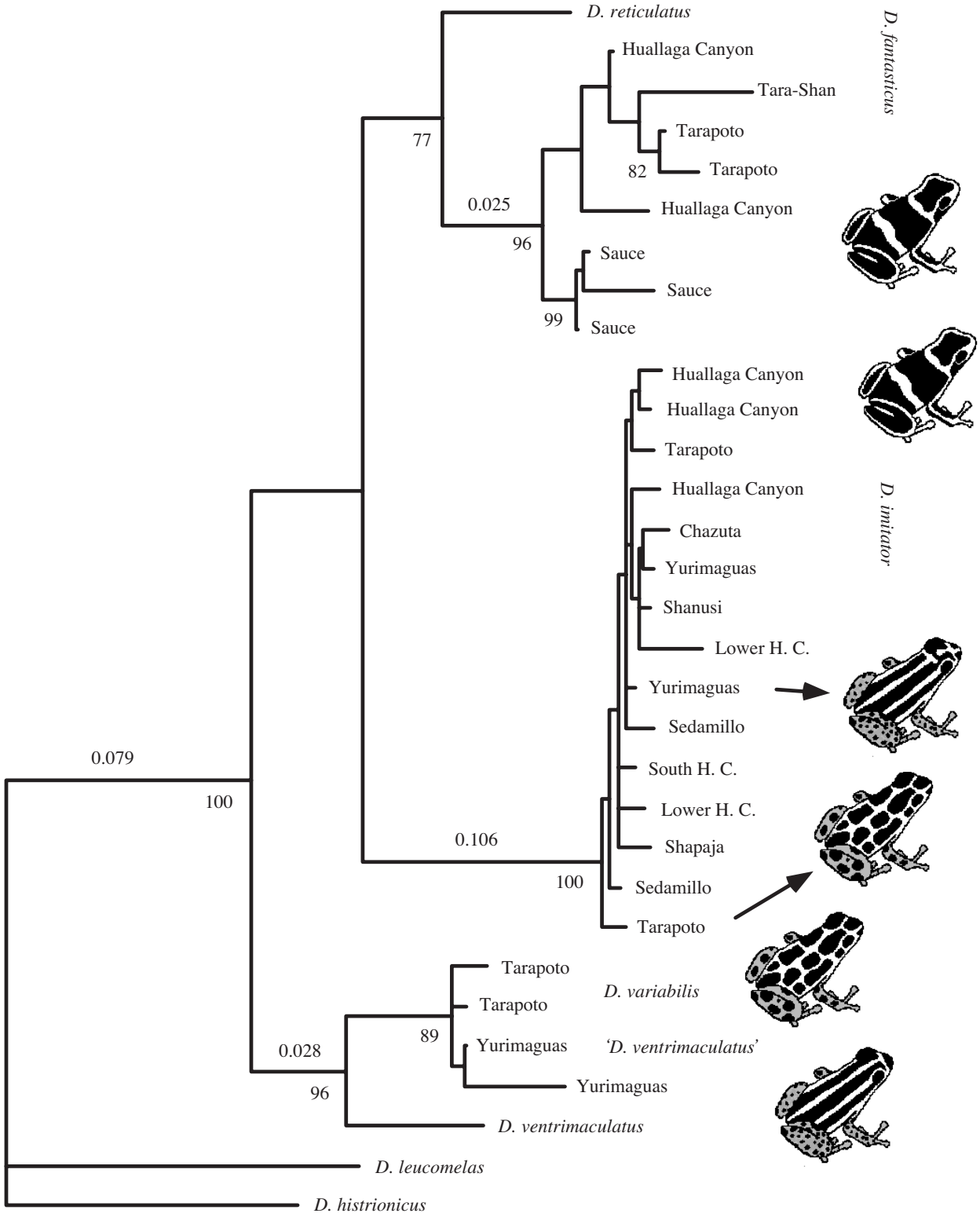

Figure 3. Phylogram showing the optimal tree topology and branch lengths from a maximum-likelihood analysis of phylogenetic relationships between the species and populations of poison frogs sampled in this study. DNA sequence data were from the cytochrome oxidase I, cytochrome *b*, 16S rRNA and 12S rRNA mitochondrial gene regions. The numbers below certain nodes represent bootstrap values for those nodes from a bootstrapped parsimony analysis; only values higher than 75% are shown. Numbers above selected branches indicate branch lengths calculated via maximum likelihood. Silhouettes indicate colour and pattern morphs that are convergent between populations of *Dendrobates fantasticus*, *Dendrobates variabilis*, '*Dendrobates ventrimaculatus*' and *Dendrobates imitator*. Individuals sequenced are designated by population location for the four focal species. Abbreviation: H. C., Huallaga Canyon.

Müllerian mimetic radiation in amphibians. Even though the evidence presented here is consistent with the mimicry hypothesis, this study does not demonstrate conclusively that mimicry caused the diversification of colour and pattern within *D. imitator*. Further work on this system is necessary to demonstrate mimicry beyond reasonable doubt. In particular, it is important to demonstrate natural selection against alternative morphs

Table 1. Kishino–Hasegawa tests of *a priori* and Shimodaira–Hasegawa tests of *a posteriori* phylogenetic hypotheses (see § 3). Shimodaira–Hasegawa tests were carried out using resampling estimated log-likelihood bootstrapping (one-tailed test) with 1000 replicates.

hypothesis (tree)	−ln likelihood	diff −ln likelihood	s.d. (diff)	*t*	*p*
Kishino–Hasegawa tests	(*a priori* hypotheses)				
D. imitator–*D. imitator*	8696.6997				
D. imitator–*D. fantasticus*	10107.6200	1410.9204	92.4328	15.2643	< 0.0001
D. imitator–*D. variabilis*	9864.7073	1168.0077	74.2787	15.7247	< 0.0001
D. imitator–'*D. ventrimaculatus*'	9946.8889	1250.1892	74.7527	16.7243	< 0.0001
Shimodaira–Hasegawa tests	(*a posteriori* hypotheses)				
unconstrained	7385.3775				
D. imitator–*D. fantasticus*	7739.6523	354.2748			< 0.001
D. imitator–*D. variabilis*	7838.1388	452.7613			< 0.001
D. imitator–'*D. ventrimaculatus*'	7856.3723	470.9947			< 0.001

in specific locations. This has been done experimentally in some butterfly mimicry systems (e.g. Kapan 2001).

With respect to the direction of mimicry, it could be argued that it is equally likely that *D. fantasticus* is mimicking *D. imitator* in the Huallaga Canyon, as the former species also displays dramatic geographical variation in colour and pattern. We consider this to be unlikely for several reasons. First, the genetic distances between populations of *D. fantasticus* from Huallaga Canyon and Tarapoto were higher ($n=4$, $\bar{x}=0.029$) than those between *D. imitator* populations from these locations ($n=6$, $\bar{x}=0.015$). Hence, the *D. imitator* populations have probably diverged more recently that the *D. fantasticus* populations, suggesting that *D. imitator* is more likely to be the mimic. Second, *D. fantasticus* populations from the other side of the Huallaga River near Sauce (where *D. imitator* do not occur) have the same colour and pattern as those in the Huallaga Canyon. This suggests that this colour and pattern is relatively ancient in these populations of *D. fantasticus*. Finally, the unique colour and pattern of *D. fantasticus* near Tarapoto suggests that it gets enough protection alone, and so is likely to be a model.

Dendrobates imitator also mimics *D. variabilis* near Tarapoto. *Dendrobates fantasticus* occurs sympatrically with these two species, but resembles neither of them. Theory predicts that mimics should converge on a more abundant model (Mallet & Joron 1999). Recently, this prediction has received empirical support (Joron *et al*. 2001). Our field observations suggest that *D. variabilis* is more common than *D. fantasticus* in the mountains near Tarapoto, which is consistent with this argument.

Dendrobates imitator also resembles poison frogs inhabiting the lowlands near Yurimaguas ('*D. ventrimaculatus*'). The close relationship between '*D. ventrimaculatus*' and *D. variabilis* suggests that they are sister taxa, if not conspecific (figure 3). Moreover, both of these taxa are closely related to *D. ventrimaculatus* (figure 3).

Again, one could ask which species is mimicking which, but the high genetic distance between *D. variabilis* from Tarapoto and the '*D. ventrimaculatus*' population from Yurimaguas ($n=4$, $\bar{x}=0.023$) relative to the genetic distance between populations of *D. imitator* from the same localities ($n=4$, $\bar{x}=0.013$) suggests that the *D. imitator* populations have diverged more recently, and are more likely to be the mimics.

Obviously, many questions remain concerning this apparent mimetic radiation. Our phylogenetic analysis suggests that *D. imitator* has undergone a relatively recent geographical radiation, and this species appears to occupy a wider variety of habitats than either *D. fantasticus* or *D. variabilis*. This may indicate that *D. imitator* is able to exploit a wider range of microhabitats relative to the models. The occupation of a generalist niche could make *D. imitator* particularly likely to undergo a mimetic radiation (Joron *et al*. 2001).

The agents (predators) that may be causing selection to favour mimicry are unknown. Canopy and understorey predatory birds are likely candidates because of their generally keen visual systems, but this remains speculative at present. The causes of diversity in coloration among populations of the models (particularly *D. fantasticus*) are not clear. Across the poison-frog family there is a significant relationship between toxicity and coloration (Summers & Clough 2001). However, this may not be relevant to the diversification within *D. fantasticus* because the different morphs of this species appear equally impressive in their potential to advertise toxicity. Other species have undergone similarly dramatic radiations in colour and pattern without any apparent link to changes in toxicity (Daly & Myers 1967; Summers *et al*. 1997; Gray 2000). The reasons for these radiations are not well understood, although sexual selection and social selection have been proposed as possible explanations (Summers *et al*. 1997, 1999*b*; Gray 2000). In butterflies, ecological divergence, biotic drift and genetic drift have been proposed as forces that could potentially cause divergence between populations (Mallet & Joron 1999). These factors could potentially influence divergence between poison-frog populations as well. Coevolutionary chase of models by 'pseudo-Batesian' mimics has also been proposed as an explanation for polymorphism and diversification in toxic butterflies (Speed & Turner 1999). This explanation is unlikely to apply to the species described here because *D. imitator* is probably more toxic than its models (Schulte 2001).

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.