The evolution of coloration and toxicity in the poison frog family (Dendrobatidae)

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The poison frogs (family Dendrobatidae) are terrestrial anuran amphibians displaying a wide range of coloration and toxicity. These frogs generally have been considered to be aposematic, but relatively little research has been carried out to test the predictions of this hypothesis. Here we use a comparative approach to test one prediction of the hypothesis of aposematism: that coloration will evolve in tandem with toxicity. Recently, we developed a phylogenetic hypothesis of the evolutionary relationships among representative species of poison frogs, using sequences from three regions of mitochondrial DNA. In our analysis, we use that DNA-based phylogeny and comparative analysis of independent contrasts to investigate the correlation between coloration and toxicity in the poison frog family (Dendrobatidae). Information on the toxicity of different species was obtained from the literature. Two different measures of the brightness and extent of coloration were used. (*i*) Twenty-four human observers were asked to rank different photos of each different species in the analysis in terms of contrast to a leaf-littered background. (*ii*) Color photos of each species were scanned into a computer and a computer program was used to obtain a measure of the contrast of the colors of each species relative to a leaf-littered background. Comparative analyses of the results were carried out with two different models of character evolution: gradual change, with branch lengths proportional to the amount of genetic change, and punctuational change, with all change being associated with speciation events. Comparative analysis using either method or model indicated a significant correlation between the evolution of toxicity and coloration across this family. These results are consistent with the hypothesis that coloration in this group is aposematic.

aposematism | phylogeny | amphibian

Aposematism (warning coloration) occurs when conspicuous appearance (particularly coloration) functions to advertise unprofitability (unpalatability, toxicity, or ability to resist or escape predation) to predators (1). The evolution of aposematism has been the subject of considerable debate (2, 3). A number of selective factors have been proposed to favor aposematism, including kin selection (4), individual selection (5), and ultra-selfish gene selection (6).

Theoretical models of the evolution of aposematism predict that conspicuous coloration will become correlated with unprofitability over evolutionary time (1, 7). This evolutionary correlation should be reflected in variation across species: unprofitable species will tend to be more brightly colored than profitable species, *ceteris paribus* (8). Studies of a variety of organisms have pointed to a correlation between measures of unprofitability (such as unpalatability or toxicity) and bright coloration as evidence for aposematism [e.g., insects (8), birds (9), amphibians (10), and reptiles (11)].

Unfortunately, many of the previous investigations of the correlation between coloration and unpalatability did not control for phylogenetic affinities among the taxa compared. Phylogenetic relationships can profoundly bias the correlation between characters in comparative analyses, and should be controlled for with specific methods (12). Recently, studies of aposematism have begun to use these kinds of comparative methods (12, 13). Here we present a comparative analysis of the evolution of coloration and toxicity in the poison frog family (Dendrobatidae) by using a recently derived hypothesis of evolutionary relationships based on mtDNA sequence variation (14).

The poison frogs are probably best known for the bright coloration and extreme toxicity that characterizes some species in this family (15). Dendrobatids produce some of the most toxic alkaloid poisons known (16). Experiments with mice indicate that minute quantities of many of these toxins are lethal to vertebrates if they enter the bloodstream (17, 18). Experiments with both vertebrate and invertebrate predators have demonstrated the unpalatability of toxic species of *Dendrobates* and *Phyllobates* (18, 19).

These studies suggest that the bright coloration of the toxic dendrobatids serves an aposematic function, that is, it serves as warning coloration in interactions with potential predators (15). However, this hypothesis has not been tested experimentally or with modern comparative methods. The hypothesis that the evolution of higher toxicity selects for the evolution of brighter, more extensive coloration predicts that the evolution of these two characteristics will be correlated (8). The correlation between the evolution of toxicity and coloration across species can be tested by using an analysis of independent contrasts, a method that controls for the effect of phylogenetic affinities in the comparative analysis of quantitative characters (20).

The diversity of coloration and toxicity in the dendrobatids makes a comparative approach to this hypothesis feasible, given an adequate phylogeny for this group. The family Dendrobatidae is believed to be monophyletic (21), with the toxic dendrobatids forming a monophyletic clade within the family (22–24). Myers (25) placed the toxic dendrobatids into four genera: *Phyllobates*, *Dendrobates*, *Epipedobates*, and *Minyobates. Dendrobates* and *Phyllobates* were diagnosed as sister taxa based on the shared presence of a unique class of alkaloid toxins (3,5-disubstituted indolizidines), shared loss of cephalic amplexus during mating, and complete loss of the primitive oblique lateral stripe (25).

Recent analyses (14) of mtDNA sequence divergence among species within the family Dendrobatidae has provided a more detailed hypothesis of phylogenetic relationships among representative species of *Allobates*, *Epipedobates*, *Minyobates*, *Phyllobates*, and *Dendrobates* (Fig. 1).

The goal of this study was to use the hypothesis of evolutionary relationships produced by phylogenetic analysis of mtDNA sequence data and comparative analysis of independent contrasts (20) to investigate the relationship between toxicity and coloration in the Dendrobatidae in a phylogenetic context. This method allows us to disentangle associations among these traits that are caused by shared ancestry from those that are caused by tandem evolutionary change (20).

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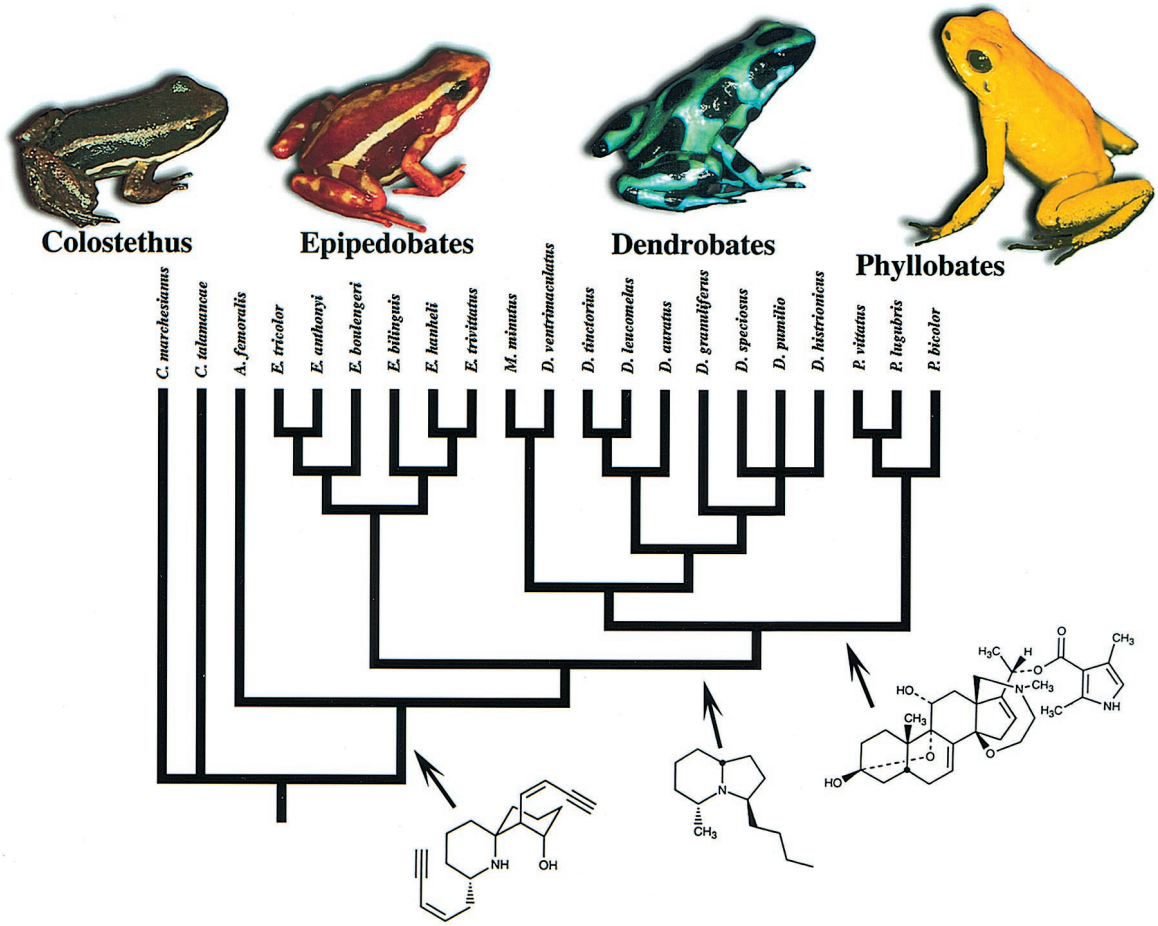

Fig. 1. Hypothesis of phylogenetic relationships among representative species in the Dendrobatidae, based on mtDNA sequences from cytochrome *b*, 12S rRNA, and 16S rRNA gene regions (14). The figure shows the relationships among 21 species for which data on toxicity and coloration were available (Table 1) and is derived from a more extensive tree including a total of 27 representative species (14). Photos across the top of the figure show representative members of the following genera: *Colostethus* (*C. talamancae*), *Epipedobates* (*E. tricolor*), *Dendrobates* (*D. auratus*), and *Phyllobates* (*P. bicolor*). Structural diagrams along the bottom show the inferred (by means of parsimony) origins of some representative toxins. From left to right the toxins are a histrionicotoxin, a 3,5-disubstituted indolizidine, and a batrachotoxin.

Materials and Methods

The aim of this study was to investigate the evolution of coloration and toxicity in representative species of poison frogs. Therefore, we acquired data on coloration and on toxicity for each of the species listed in Table 1.

Data on coloration was obtained from ratings of color photographs of species in the phylogeny. Ratings were obtained from a total of 24 human observers. The observers were not familiar with the frogs and did not know the levels of toxicity in the different species. A photo (taken with a flash) showing the dorsum and side of each of 21 species was rated for brightness and extent of coloration in a composite measure. The mean measure of coloration across observers was used for each species (Table 1). Naturally, it would be preferable to use potential predators rather than humans to rate the coloration of the frogs, but this method was not feasible. Birds are likely to represent an important class of predators for small vertebrates living on the forest floor, including anurans (26). Recent research suggests that animals that appear colorful to humans are also likely to appear colorful to birds (27).

An independent analysis of coloration was done by using scanned color photos (taken with a flash) of each species in the phylogeny. By using the pixel sampler in the computer program Adobe PHOTOSHOP (28), the brightness of color for each of the three major color hues (red, blue, and green) was sampled at 10 different points on each differently colored region of each species. The proportion of the frog that each colored region comprised was measured with the National Institutes of Health IMAGE program (29). A similar analysis was carried out on photos of leaf litter (taken with a flash), which allowed us to contrast the overall brightness of coloration of each species of frog relative to a leaf-littered background. Overall coloration was quantified as the contrast (to leaf litter) for each colored region multiplied by the proportion of the frog that each colored region comprised. The data from the computer ratings are shown in Table 1. The scale of variation in coloration was 1–10 for both the observer and computer-rated analyses.

Information on the toxicity of different species was taken from the work of Daly *et al.* (30). Overall toxicity was scored for three different attributes: diversity, quantity, and lethality. Diversity refers to the number of different toxins found in skin extracts from each species. Quantity refers to the amount of alkaloids detected in 100 mg of skin: 3 = > 150 μg/mg; 2 = 50 − 150 μg/mg; 1 = 1 − 50 μg/mg; and 0 = no alkaloids detected (30). Data on both diversity and quantity were averaged across populations when more than one population of a species was sampled.

Data on the lethality of all of the different dendrobatid toxins has not yet been published, but the evidence indicates that the batrachotoxins are the most toxic alkaloids found in these frogs

Table 1. Data used for the comparative analysis of toxicity and coloration

Genus	Species	Diversity	Quantity	Lethality	Total toxicity	Coloration (observer)	Coloration (computer)
Allobates	*femoralis*	0.17	0.167	1	1.2	3.8	0.3
Colostethus	*marchesianus*	0	0	0	0	2.2	0.2
Colostethus	*talamancae*	0	0	0	0	1.1	0.7
Dendrobates	*auratus*	16.8	2.556	2	6.2	5.9	4.8
Dendrobates	*granuliferus*	17	2	2	5.7	6.7	3.4
Dendrobates	*histrionicus*	18.1	2.571	2	6.4	7.1	2.9
Dendrobates	*leucomelas*	7	1	2	3.7	7.1	2.6
Dendrobates	*pumilio*	16	2.25	2	5.9	7.8	4.4
Dendrobates	*speciosus*	18.5	2.75	2	6.6	7.9	4.0
Dendrobates	*tinctorius*	10	2	2	5	7.8	3.7
Dendrobates	*ventrimaculatus*	12.5	2	2	5.3	6.1	3.2
Epipedobates	*anthonyi*	2	1	1	2.2	4.8	1.7
Epipedobates	*bilinguis*	6	1.5	1	3.1	4.8	1.3
Epipedobates	*boulengeri*	12	1	1	3.2	1.6	0.5
Epipedobates	*hahneli*	6	2	1	3.6	3.3	1.1
Epipedobates	*tricolor*	13	3	1	5.3	5.6	2.0
Epipedobates	*trivittatus*	8	2.667	1	4.5	5.7	2.9
Minyobates	*minutus*	7	1.2	1	2.9	3.7	1.0
Phyllobates	*bicolor*	12	3	3	7.2	7.9	8.5
Phyllobates	*lugubris*	1.7	0.67	3	3.8	3.7	1.7
Phyllobates	*vittatus*	2	1	3	4.2	4.3	4.5

Total toxicity was a composite measurement of the diversity, quantity, and toxicity of the toxins found in each species. Coloration was scored with computer software and by human observers (see text).

[and one of the most toxic alkaloids on earth (16)]. The second most toxic class of alkaloids produced by these frogs seems to be the pumiliotoxins classes A and B, which are substantially more lethal than the other major classes of alkaloids identified in these frogs (18). The batrachotoxins are found only in the members of the genus *Phyllobates* (16). Classes A and B pumiliotoxins are found as major components of the alkaloid profile mainly in the genus *Dendrobates* (13 of 14 species analyzed) and rarely in the genus *Epipedobates* (1 of 8 species analyzed; ref. 30). *Phyllobates* and *Dendrobates* also share a class of toxins, the 3,5-disubstituted indolizidines (31), which are not found in *Epipedobates*, *Minyobates*, *Allobates*, or *Colostethus*.

Hence the category "lethality" attempts to quantify consistent differences among genera in the toxicity of the major components of their skin-alkaloid profiles. This measure was scored on a four-point scale, with *Phyllobates* receiving the highest score with 3, *Dendrobates* next with 2, *Epipedobates* and *Minyobates* next with 1, and *Colostethus* [most members of which showed little or no toxicity (31)] was given the lowest rating of 0.

These measures were combined for an overall toxicity score as follows (Table 1): (0.1)(diversity) + (quantity) + (lethality). The scale on which diversity was measured was $\approx$10 times larger than that of the other factors (Table 1), thus down-weighting diversity relative to quantity and lethality actually assigns approximately equal weight to each category in the final composite measure of overall toxicity. The exact contribution of each of these three aspects of toxicity (diversity, quantity, and lethality) to overall toxicity is not known, but tests with mice suggest that quantity contributes to the overall lethality of different species of poison frogs [survival time for mice injected with toxic alkaloids from poison frogs is dosage-dependent (17)]. Tests with mice also indicate that some toxins that are unique or major alkaloid components in only certain taxa (such as batrachotoxin) are much more lethal than others (18). The contribution of toxin diversity to overall toxicity is not known but a more diverse toxin profile is likely to increase both lethality and unpalatability, *ceteris paribus*.

Data on toxicity refers to average levels for specific species (not the specific individuals scored for color in the photographs as described above). Data on toxicity were not available for *Epipedobates boulengeri* but we assigned toxicity to it according to that of its closest relative, *Epipedobates espinosai* [these two species were previously considered conspecific (23)]. *E. espinosai* is more brightly colored than *E. boulengeri* (23) and shows moderate toxicity (30). Hence, this assignation is likely to be conservative with respect to the hypothesis that coloration and toxicity coevolve. Some researchers have classified *Epipedobates tricolor* and *Epipedobates anthonyi* as members of the same species (32). Hence, we have not included a contrast between these two species or populations in our analysis, but we have placed the data for both of them in the overall analysis to compare both with the other species in the phylogeny. Populations of two species sampled by Daly *et al.* (30) have since been assigned to different species names: *Epipedobates hahneli* for *Epipedobates pictus* and *Epipedobates bilinguis* for *Epipedobates parvulus* (14). The names *E. hahneli* and *E. bilinguis* are used in this article.

The phylogenetic analysis was carried out with sequence data from portions of the 16S rRNA, 12S rRNA, and cytochrome *b* regions of mtDNA from each species. The combination of 16S rRNA, 12S rRNA, and cytochrome *b* sequence fragments provided a total of 1,198 bases for analysis, of which 589 exhibited 1 or more state changes (14).

Phylogenetic analysis [using the program PAUP (33)] produced a single most parsimonious tree for the ingroup taxa, but the outgroup species (*Colostethus talamancae* and *Colostethus marchesianus*) formed a basal polytomy with the ingroup (Fig. 1). This hypothesis of phylogenetic relationships had a consistency index of 0.48 and a retention index of 0.62, with a total tree length of 1,823 (14). The results of the analysis were largely in agreement with the generic relationships proposed by Myers (25), with the exception that *Allobates femoralis* was placed outside of the other toxic dendrobatids, and *Minyobates minutus* was placed as the sister species to a species within the genus *Dendrobates* (*Dendrobates ventrimaculatus*). The results also were congruent with a recent hypothesis of phylogenetic relationships within the genus *Dendrobate*s based on a different mtDNA data set (34), with the exception of the relationships among *Dendrobates*

Table 2. Statistics for the regression of the standardized contrasts of coloration on toxicity, under the punctuational and gradual models of evolutionary change, using observer- and computer-rated estimates of coloration

Model	Rating	Contrasts	Tandem	R^2	*F* value	*t* value	*P* value
Punctuational	Observer	17	14	0.51	16.43	4.10	0.001
Gradual	Observer	17	13	0.38	9.83	3.14	0.006
Punctuational	Computer	17	15	0.83	78.89	8.88	0.000
Gradual	Computer	17	15	0.71	38.27	6.19	0.000

Tandem refers to the number of contrasts in which toxicity and coloration evolved in the same direction.

histrionicus, *Dendrobates pumilio*, and *Dendrobates speciosus*. The relationships among these species were not supported strongly in our phylogenetic analysis (14), thus this clade was collapsed into a polytomy for the comparative analysis (Fig. 1).

The phylogenetic tree derived from mtDNA data, together with data on coloration and toxicity, were used to generate phylogenetically independent contrasts by using the computer program CAIC (35). This method enabled us to investigate the correlation between evolutionary change in coloration and toxicity. Recent studies suggest that methods that reconstruct ancestral states can be subject to substantial levels of uncertainty (36). However, this type of uncertainty has a stronger effect on the results of methods that rely on the exact reconstruction of each ancestral state than on correlative comparative methods (such as the comparative analysis of independent contrasts), in which ancestral-state reconstruction plays a secondary role (36). Recent research indicates that the comparative analysis of independent contrasts accurately reconstructs correlations between evolutionary events, even when exact ancestral states are not accurately reconstructed (37). Given a general lack of knowledge about modes of character evolution, the most conservative approach when using comparative analysis of independent contrasts is to try several distinct models of character evolution to determine whether different assumptions substantially alter the results of the analysis (38). Here we have used two maximally distinctive models of character evolution, both of which can be implemented by CAIC, a punctuational model in which all change occurs at speciation (cladogenetic) events, and a gradual change model, in which the amount of change is proportional to the branch length. Branch lengths were calculated on the basis of the amount of genetic change occurring on each branch. Genetic change was quantified as the genetic distance based on sequence divergence from our previous analysis of mtDNA sequences (14), calculated with the Kimura (39) two-parameter model.

The relationships between the standardized contrasts produced by CAIC were tested for statistical significance by regression through the origin by using coloration as the dependent variable. For each regression, the residuals were tested for significant deviations from normality with a Kolmogorov–Smirnov test, and plots of the residuals vs. the fitted values were examined for symmetry and homogeneity. Statistical analyses were carried out with the computer program STATVIEW (40).

Results

Overall toxicity scores varied substantially among species (Table 1). Both measures of coloration also showed substantial variation among species (Table 1). Fig. 1 shows the phylogenetic relationships among the species analyzed in this study. The data on toxicity and coloration were used in combination with this phylogeny to carry out a comparative analysis of independent contrasts for each combination of color-scoring method and mode of character evolution. Table 2 shows the regression statistics for each analysis. Fig. 2 shows the plots of the regression of standardized contrasts of coloration on toxicity for each analysis. There was a significant association between the evolution of coloration (as rated by human observers) and the evolution of overall toxicity (as quantified by toxin diversity, quantity, and lethality). This was the case assuming either a punctuated or a gradual mode of evolution for these traits (Table 2; Fig. 2 *a* and *b*). Computer rankings of coloration showed a stronger evolutionary association with toxicity than the observer ratings of coloration. Again, this was the case assuming either a punctuated or a gradual mode of evolution (Table 2; Fig. 2 *c* and *d*). Hence, our results indicate that coloration has evolved in tandem with toxicity in the poison frogs of the family Dendrobatidae.

Discussion

The evolution of aposematism has received extensive attention in the literature (6). However, few comparative analyses controlling for phylogenetic effects have been carried out to test a basic prediction of the theory of aposematism, that more toxic species will advertise their toxicity more conspicuously, with brighter, more extensive coloration. The poison frogs provide a good opportunity to test this prediction because of the wide variation in coloration and toxicity within this family. Our phylogenetic analysis (14) provides information on evolutionary relationships that is crucial for comparative analysis (20). The measures of both toxicity and coloration used in this study were fairly crude. Despite this lack of precision, which could obscure any relationship between these two variables, variation in toxicity explained a significant amount of variation in coloration under each combination of measurement technique and model of evolutionary change. It is certainly possible that other selective factors have influenced the evolution of coloration in these frogs. Nevertheless, our results suggest a substantial role for toxicity in the evolution of bright coloration in the poison frogs.

Our results do not guarantee that comparisons of coloration and toxicity among populations within species also will reveal a positive correlation between the evolution of coloration and toxicity. For example, there does not seem to be a correlation between the brightness of coloration and toxicity among divergent color morphs from different populations of *Dendrobates pumilio* (17) in the Bocas del Toro Archipelago, Panama (although this study did not control for phylogenetic relationships among populations, because these are not known). In this case, other factors are likely to be influencing the evolution of color, independent of toxicity (41).

Variation in color or pattern among populations of poison frogs is not uncommon, although this typically consists of variation in color hue and pattern, not presence or absence of coloration (15, 42). There is also considerable variation in toxin profiles among populations of some species, which may be associated with dietary factors (31).

Recent results have demonstrated that some species of poison frogs accumulate and sequester some of their skin toxins from dietary sources (31). Dendrobatid frogs can accumulate a variety of alkaloid toxins added to their diet as supplements (43). Dendrobatid frogs raised on a diet of insects derived from forest leaf litter accumulate toxins in their skin, whereas frogs raised on fruit flies do not (44). Possible sources of dendrobatid toxins

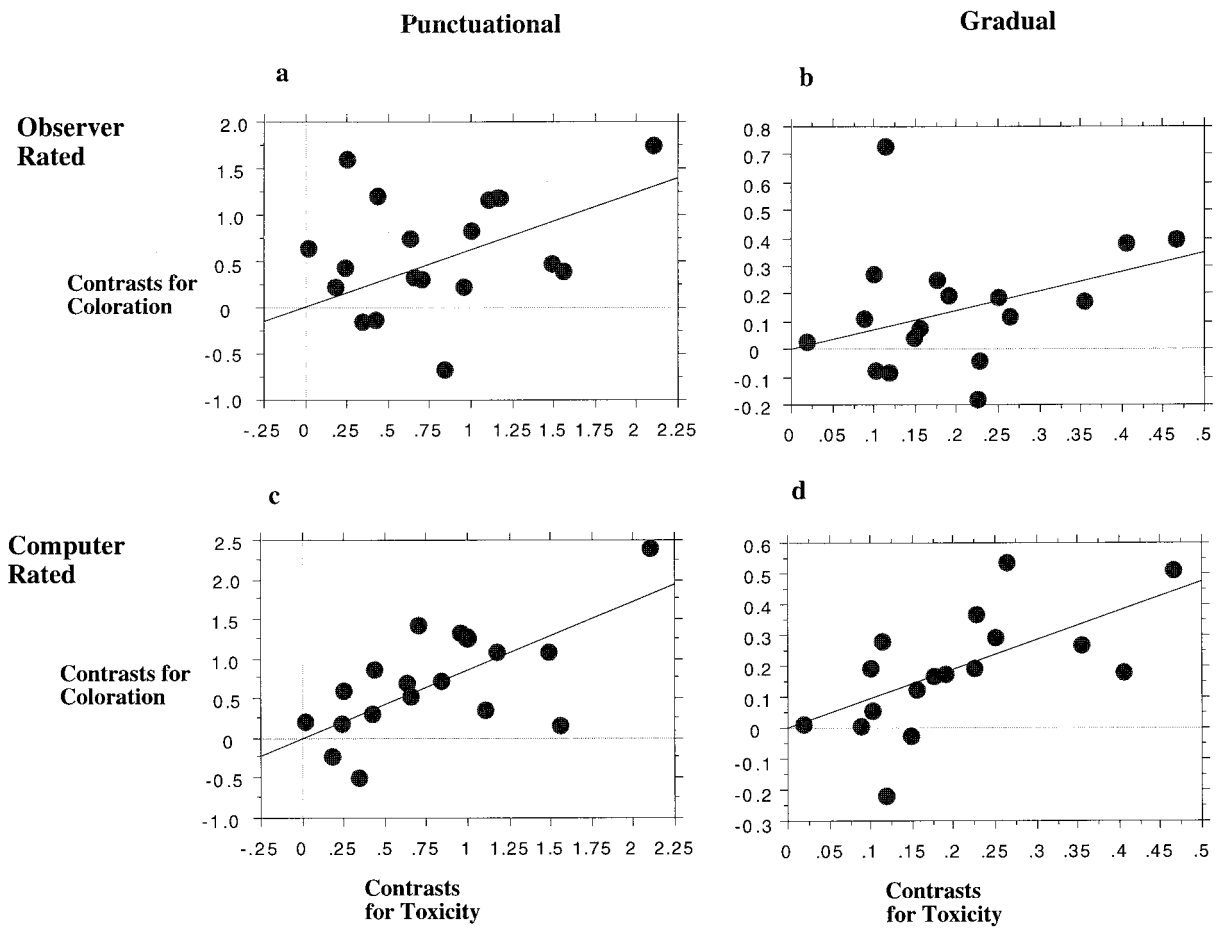

Fig. 2. Regressions of standardized contrasts for coloration on standardized contrasts for toxicity across the poison frogs. (*a*) Coloration as measured by human rates vs. toxicity, using a punctuational model of evolutionary change in each character. (*b*) Coloration as measured by human raters vs. toxicity, using a gradual model of evolutionary change. (*c*) Coloration as measured with a computer-based analysis vs. toxicity, using a punctuational model of evolutionary change. (*d*) Coloration as measured with a computer-based analysis vs. toxicity, using a gradual model of evolutionary change.

include ants (pyrrolizidines and indolizidines), beetles (coccinellines), and small millipedes (pyrrolizidine oximes; ref. 45). Comparative studies suggest a possible correlation between a tendency to consume a high proportion of ants by dendrobatid frogs and their levels of toxicity (46, 47).

These results do not imply that differences among species in toxin profiles are not genetically controlled (31). Between species, differences are higher than within species variation, and different species have consistently different toxin profiles (31). Many species of *Dendrobates* are sympatric with species of *Phyllobates*, yet all species of *Phyllobates* have batrachotoxins, whereas species of *Dendrobates* do not (31). Toxin profiles differ significantly even between sympatric populations of closely related species, such as *D. pumilio* and *Dendrobates granuliferus* (31). Hence, genetic differences are likely to control the ability of different species to absorb and sequester specific toxins from their diet (31).

Also, many of the compounds found in poison frog skin have not been found in either plants or insects (e.g., decahydroquinolines, gephyrotoxins, histrionicotoxins, pumiliotoxins, allopumiliotoxins, homopumiliotoxins, 5,8-disubstituted indolizidines, epibatidines, and batrachotoxins). This observation suggests that some of these compounds may in fact be synthesized in the skin glands of the frogs (48). Differences among species in such synthetic abilities would presumably be genetic in origin.

Finally, natural selection could favor a correlation between coloration and toxicity even if differences in toxicity were entirely a function of dietary differences among species. If differences among species in dietary preferences or predatory capabilities are heritable, then natural selection could act to favor brighter coloration in species that consistently have preferences for or access to prey with more or more potent toxins.

The results presented here indicate that toxicity and coloration have evolved in tandem in the poison frog family. This evolutionary correlation is consistent with the hypothesis of aposematism as an explanation for the evolution of bright coloration in this family.

However, several caveats are in order. First, correlation does not demonstrate causation, and experiments on predator learning in response to interactions with bright and dull toxic individuals are required to further test the hypothesis of aposematism in the poison frogs (6). Investigating the effects of experimental manipulations of coloration on survival in natural populations will also be critical in testing the hypothesis of aposematism (49).

Several alternative hypotheses for a correlation between coloration and toxicity have been suggested (6). Strong sexual selection may cause selection for bright displays in males, females, or both sexes (50). In turn, this coloration could select for increased unpalatability in response to increased predation rates (6). This hypothesis seems unlikely to explain the evolution of bright coloration in dendrobatid frogs [as opposed to the evolution of variation in color hue and pattern among populations (41)], because strong sexual selection is common in cryptic

species of the genus *Colostethus* just as it is in toxic brightly colored species, such as those of the genus *Dendrobates* (51).

Bright coloration also may be associated with thermoregulation (again favoring selection for unpalatability after the fact). This hypothesis is also unlikely in the toxic dendrobatids, because their bright colors and patterns do not conform to expectations for thermoregulatory coloration based on functional considerations (52). Furthermore, the toxic dendrobatids frequently occur microsympatrically with nontoxic cryptically colored relatives from the genus *Colostethus* (51). Observations indicate that members of the genus *Colostethus* spend as much time exposed on the forest floor as their toxic counterparts (51), suggesting that bright coloration is not required for effective thermoregulation by these frogs. However, although consideration of the biology of the poison frogs suggests these scenarios are less likely, they are consistent with the evidence presented in this article.

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