

Multiple, recurring origins of aposematism and diet specialization in poison frogs

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Aposematism is the association, in a prey organism, of the presence of a warning signal with unprofitability to predators. The origin of aposematism is puzzling, because of its predicted low probability of establishment in a population due to the prey's increased conspicuousness. Aposematism is a widespread trait in invertebrate taxa, but, in vertebrates, it is mostly evident in amphibians, reptiles, and fishes. Poison frogs (Dendrobatidae) are one of the most well known examples of the co-occurrence of warning coloration and toxicity. This monophyletic group of mostly diurnal leaf-litter Neotropical anurans has both toxic/colorful and palatable/cryptic species. Previous studies suggested a single origin of toxicity and warning coloration, dividing the family in two discrete groups of primitively cryptic and more derived aposematic frogs. Recent molecular phylogenetic analyses using mostly aposematic taxa supported this conclusion and proposed a single tandem origin of toxicity and conspicuous warning coloration. By using expanded taxon and character sampling, we reexamined the phylogenetic correlation between the origins of toxicity and warning coloration. At least four or five independent origins of aposematism have occurred within poison frogs; by using simulations, we rejected hypotheses of one, two, or three origins of aposematism ($P < 0.002$). We also found that diet specialization is linked with the evolution of aposematism. Specialization on prey, such as ants and termites, may have evolved independently at least two times.

Warning signals may inform a predator that the intended prey is toxic, unpalatable, or generally not worth the predator's effort. The association of unprofitability with a warning signal, such as bright or conspicuous coloration, is known as aposematism. Its evolutionary origin has posed a conundrum since the time of Wallace and Darwin (1). Although aposematism evolves as a predator deterrent, its chance of establishment in a population is predicted to be low, because it would lead to an increased probability of predation (2). Aposematism exists in many invertebrates, fishes, amphibians, snakes, and birds (3). Models proposed to explain the origin of aposematism (e.g., individual selection versus kin selection, gregariousness, green-beard selection; refs. 4–7) treat trait evolution at only the population level. In contrast, a phylogenetic perspective can provide evidence for the likelihood of historical patterns of trait evolution (e.g., does toxicity always evolve before conspicuousness?), but this approach has rarely been examined (4, 8, 9).

Well supported and well sampled phylogenies are fundamental for comparative biology, and reliable inferences should likely be derived from them (8, 10). Ancestral character states, and their order of appearance (i.e., character mapping), can be mistakenly reconstructed if taxon sampling is not comprehensive (11). Although most analyses of aposematism perform tests for predator deterrence based on hypotheses of current utility only, aposematism can be defined within a historical context as the consecutive or prior occurrence of unpalatability, relative to conspicuousness (8). The addition of a phylogenetic framework in these tests would facilitate the identification of the sequences of evolutionary transformations in these traits.

Poison frogs (Dendrobatidae) are a well supported monophyletic group (± 210 species) distributed in tropical South and Central America (12). The family includes both aposematic and cryptic species, all of which are diurnal, with *Aromobates nocturnus* being the one exception (13). Some species (primarily *Dendrobates*, *Phyllobates*, and *Epipedobates*) are brightly colored and possess toxic, lipophilic skin alkaloids (14). Some of these substances are of biomedical importance (15), and their source is probably dietary (16). Other species (e.g., *Colostethus*, *Mannophryne*, and *Nephelobates*) are cryptic and nontoxic, lacking lipophilic skin alkaloids, as far as is known (13, 17, 18). Based on the assumption that structurally complex biochemical compounds are difficult to evolve, the possession of alkaloids was believed to have originated once in dendrobatids (13). Phylogenetic analyses of characters other than DNA (13, 19) also proposed a single origin of toxicity. These findings were supported by an analysis of DNA sequences (20), although other smaller datasets (21) had suggested the possibility of convergence. Here we show, based on a more comprehensive taxon sample, that the association of conspicuous bright coloration and toxicity appeared not once, but several times, within poison frogs.

Aposematic species (*Dendrobates*, *Phyllobates*, and some *Epipedobates*) eat mostly ants, termites, and mites (22–24). Some *Dendrobates* exclusively eat ants or mites and reject other available prey (22). The majority of cryptic species (*Colostethus*) eat diverse prey (24), mostly everything available that is the right size. Natural history and ecological studies suggest that higher degrees of toxicity and toxin diversity are directly associated with a specialized diet (16), which has been assumed to have evolved only once within dendrobatids (22, 24). Here we present evidence that diet specialization has occurred more than once, and is tightly associated with the multiple origins of conspicuousness and toxicity.

Materials and Methods

Phylogeny Estimation. We sampled a broadly representative group of cryptic and aposematic dendrobatids. We sequenced 56 samples, of which six were outgroups [*Bufo variegatus* (Bufonidae), *Centrolene grandisonae* (Centrolenidae), *Pseudacris regilla* (Hylidae), *Pseudis paradoxa* (Pseudidae), *Telmatobius niger*, and *Adenomera* sp. (Leptodactylidae)]; for brevity, these samples are not shown on the phylogenetic trees]. The remaining 50 samples represented 38 species of dendrobatids from most species groups of *Colostethus*. Taxonomically, we include *Minyobates* within *Dendrobates*, and *Phobobates* in *Epipedobates* (20). These data included 2,298 characters from the entire 12S, tRNA-val, and almost all of the 16S mitochondrial genes. These data were combined with 22 dendrobatid sequences from GenBank, most of which consist of 900 bases that are completely overlapped by our data. Specimen museum numbers, collection localities, lists of primers used, and GenBank accession nos. can

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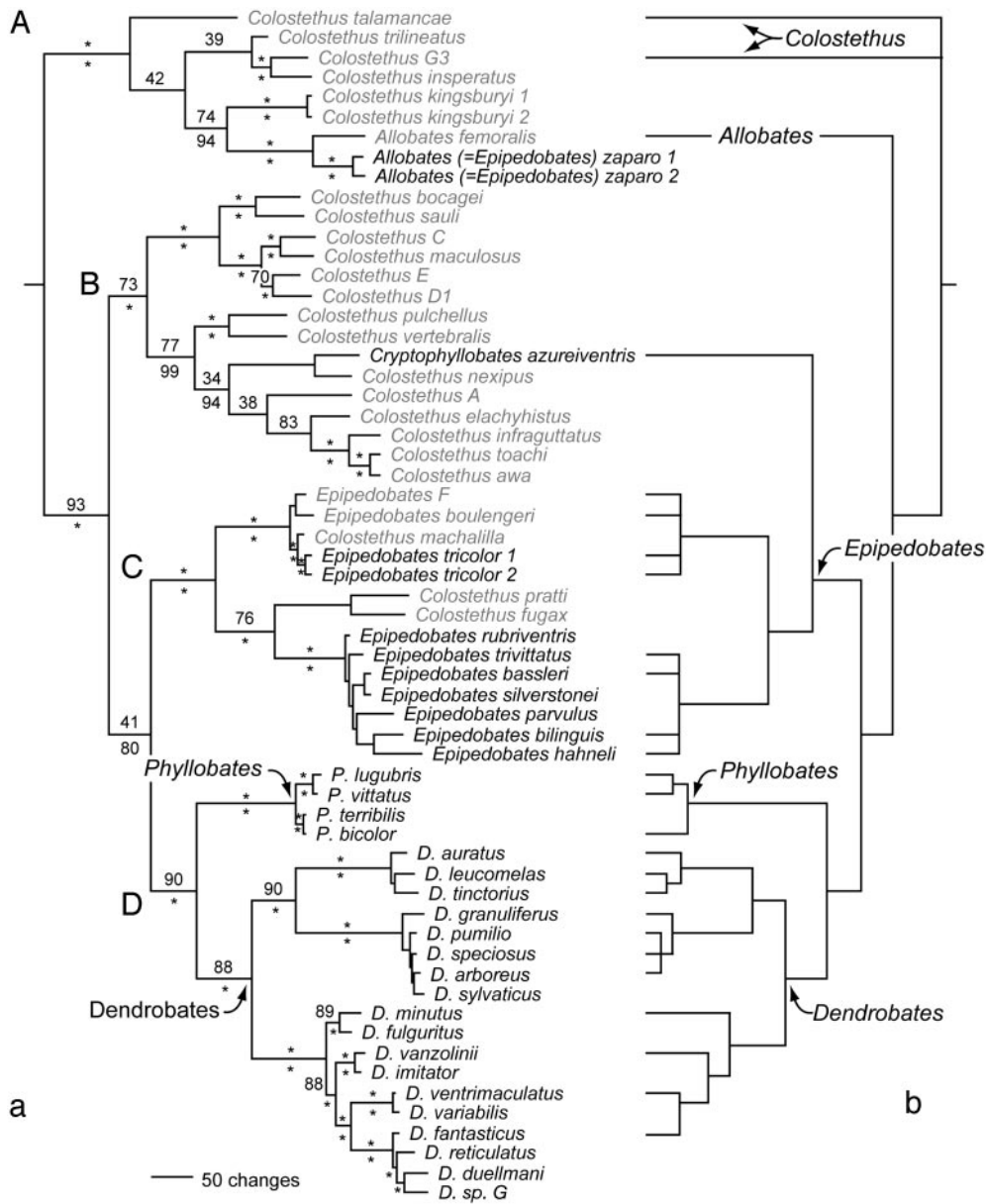


Fig. 1. (a) Phylogeny of poison frogs. Species names in black refer to conspicuous and (as far as is known) toxic species. Names in gray refer to cryptic and nontoxic species. The tree shown is based on the parsimony analysis, but the likelihood tree (Fig. 2) is almost identical. The sister-group relationship of clades C and D was recovered in all three types of analyses. In the parsimony analysis, the sister-group relationship of clades B and C was equally supported, but this topology was not the best estimate under likelihood or Bayesian analysis. Neither alternative (clade C + D vs. clade B + C) is strongly supported by bootstrap proportions or Bayesian posterior probabilities. Parsimony bootstrap proportions are above each branch, and Bayesian posterior probabilities are below. *, a value of ≥ 95 . (b) Previous molecular phylogeny of poison frogs (33). The difference between a and b is due to the degree of taxon sampling.

dobates zaparo (clade A) to *Allobates*; thus, the name is *Allobates zaparo* (new combination). However, *Colostethus* remains grossly paraphyletic; reducing the degree of polyphyly of *Epipedobates* by recognizing *Allobates* and *Cryptophyllobates* is but a transitional solution toward a classification of dendrobatids based solely on monophyletic taxa. Notwithstanding these taxonomic changes, the remaining *Epipedobates* species in clade C are not monophyletic. Resolution of this clade is crucial for the Linnean taxonomy of dendrobatids, because it contains the type species of *Epipedobates* (*tricolor*) and may also contain the type species of *Colostethus* (*latinasus*), based on morphological similarity (17). Given the current lack of molecular data on the position of *Colostethus latinasus*, a wholesale revision of poison frog taxonomy is beyond the scope of this paper.

A striking feature of the multiple origins is that they occur on different time scales, indicating recurring origins through evolutionary history. Aposematism had a single ancient origin at the base of clade D (*Dendrobates* plus *Phyllobates*) and was not lost in any descendants in this clade. Alkaloid data are available for most species in this clade, and all are toxic. The other origins of aposematism are much more recent. For example, sequence divergence (uncorrected p-distance) between the cryptically colored *Colostethus machalilla* and its toxic, bright red sister species *Epipedobates tricolor* (Fig. 2) is a mere 0.9–1.0%. The latter species is the natural source of epibatidine, an alkaloid that is an opioid analgesic (18). In contrast, divergence in the same genes is 1.7–5.7% among five well differentiated and strikingly variable and brightly colored species of the *Dendrobates pumilio*

izidines) from food supplements (37). When raised on fruit flies, captive *Phyllobates*, *Dendrobates*, and *Epipedobates tricolor* lose much of their toxicity (38, 39). Nonetheless, despite the demonstration of alkaloid sequestration, the source of the most biologically active alkaloids; i.e., batrachotoxins, histrionicotoxins, epibatidine, and others, remains unknown (18).

Based on the available information, our phylogeny demonstrates at least two, and perhaps three, independent origins of dietary specialization (Fig. 2). One origin of diet specialization is in the ancestor of clade D (*Phyllobates* and *Dendrobates*), in which all species are ant, termite, or mite specialists (24). A second origin is within clade C (*Epipedobates* and *Colostethus*), in which some species have generalist diets (including the brightly colored *E. tricolor*), but *E. parvulus* and its relatives are ant specialists (24). A possible third origin is in clade A, in which most species, including *A. femoralis*, have generalist diets. However, the limited data (40) indicate that *A. zaparo*, its brightly colored sister species (Fig. 1), eats mostly ants. Although there is a clear phylogenetic correlation between bright coloration and toxicity (as demonstrated by the correlated changes test), data sufficient to test the association of diet are lacking for most species.

Under the single-origin hypothesis, dietary specialization and foraging ecology were predicted to be key evolutionary factors in the diversification of poison frogs (22, 24, 36). Under this multiple-origins hypothesis, the evolutionary association between diet and aposematism may be more complex. Although one large clade (D) displays an ancient origin of aposematism, most of the origins are relatively recent and involve one or a few species, suggesting that this homoplasy is dynamic and recurring. The specialization on different prey types (ants, termites, or mites), which may explain the great diversity of alkaloids, suggests selection for specialization *per se* (41), rather than commitment to a particular food resource. For example, *E. tricolor* is toxic but is not specialized on ants or termites (L.A.C., unpublished data), which suggests that this species might sequester toxins from unrecognized sources, such as larger-prey items.

The association of dietary specialization and sequestration of toxic defensive compounds in aposematic organisms is not novel to frogs. For example, two unrelated lineages of aposematic papilionid butterflies sequester aristocholic acid compounds from pipevines (Aristolochiaceae) (42). *Longitarsus* leaf beetles (Chrysomelidae) have evolved the sequestration of pyrrolizidine alkaloids multiple times (43). However, dendrobatid frogs are unique among vertebrates in their recurring associations of coloration, toxicity, and diet specialization. This observation suggests an as-yet-undefined physiological mechanism in the ancestor of poison frogs that allowed sequestration of toxic compounds.

Fragmentary but exciting evidence suggests other behavioral traits that may be associated with aposematism and dietary specialization. In contrast to almost all other frogs, both cryptic and aposematic dendrobatids are diurnal, rather than nocturnal, with the apparent exception of *A. nocturnus* (13). This change to a diurnal habit, in which visual signals would be favored, may have facilitated repeated adaptive shifts toward novel foraging ecology, dietary specialization, toxicity, and bright coloration. Also, high aerobic and low anaerobic metabolic capacity have been found in the few aposematic dendrobatid species studied (44). In contrast, cryptic species of other leaf-litter frogs (*Eleutherodactylus*) that

co-occur with these dendrobatids have low aerobic and high anaerobic capacity (44), and are not dietary specialists on ants (24). This physiological trait in some dendrobatids probably favored a recurring association of these traits. More data about metabolic rate in dendrobatids are needed within this phylogenetic framework.

Ecological specialization is a widespread evolutionary outcome in many animal systems (41). It is commonly stated that a specialization should derive from a generalized, plesiomorphic trait. This finding appears to be true in the case at hand; the traits of conspicuousness, unpalatability, and narrow diet are derived from crypticity, palatability, and a generalized diet, respectively. In the phylogeny presented here, these derived traits are not statistically independent, and probably reinforce each other, promoting evolutionary specialization. The appearance of toxicity may generally precede the appearance of diet specialization and warning coloration. Evidence for this possibility comes from three cases. First, *A. nocturnus*, the putative sister species of all dendrobatids, has a noxious, mercaptan-like odor, despite the lack of alkaloids (13) and its cryptic coloration. Second, although the few *Colostethus* sampled for lipophilic alkaloids show no traces (14), *Colostethus inguinalis* has tetrodotoxin (45), a water-soluble (rather than lipophilic) toxin otherwise unknown in dendrobatids. These two cases suggest parallel but isolated origins of defense in obviously cryptic species. Third, certain species are not brightly colored, but have either flash coloration or contrasting patterns on concealed surfaces; these species also have some degree of alkaloid toxicity (*A. femoralis*, *E. boulengeri*, and its sister species, *E. sp. F*), and are closely related to brightly colored species. These species may represent microevolutionary cases of dynamic intermediate conditions between the cryptic-palatable and conspicuous-toxic extremes. However, the data are meager and much work at the population level is needed to confirm this hypothesis.

At the other extreme, all conspicuous species (*Dendrobates*, *Phyllobates*, and most *Epipedobates*) surveyed possess diverse and often abundant toxins (14); i.e., no Batesian mimics are known. Furthermore, although the degree of diet specialization among the toxic species varies, the diets of the least specialized toxic species are still narrower than those of the cryptic species (22, 24).

In summary, a more comprehensive phylogeny reveals the multiple appearances of this complex of traits (visual signals, toxicity, narrow diet, and, perhaps, higher metabolic rate), which suggests parallel and correlated evolutionary trends toward specialization. These multiple occurrences may indicate directional selection for the acquisition of toxins from dietary components, which likely led to aposematic coloration and feeding specializations.

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