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Phenotypic and Genetic Divergence in Three Species of Dart-Poison Frogs With Contrasting Parental Behavior

K. Summers, E. Bermingham, L. Weigt, S. McCafferty, and L. Dahlstrom

Why some species exhibit remarkable variation among populations while closely related species are relatively uniform remains unclear. The strawberry dart-poison frog (*Dendrobates pumilio*) exhibits spectacular color and pattern polmorphism among populations in the Bocas del Toro archipelago of Panama. In contrast, two other sympatric species of dart-poison frog, *Phyllobates lugubris* and *Minyobates* sp., show little color or pattern variation among island populations. Here we demonstrate that the color and pattern variation among populations of *D. pumilio* is not matched by higher levels of mitochondrial DNA sequence divergence relative to *P. lugubris* or *Minyobates* sp. Thus, neutral divergence in allopatry is unlikely to have caused the geographical differences observed in *D. pumilio*. We suggest that strong sexual selection associated with female parental care in *D. pumilio*, which contrasts the male parental care of *P. lugubris* and *Minyobates* sp., may have driven divergence in coloration and pattern in *D. pumilio*.

The causes of pronounced contrasts in coloration, pattern, and other forms of ornamentation among populations or closely related species of some animals are widely debated (Mayr 1963; West-Eberhard 1983). Often this variation seems arbitrary with respect to the environment (Dominey 1984; West-Eberhard 1983). Populations of the strawberry dart-poison frog (*Dendrobates pumilio*) are extremely differentiated (Daly and Myers 1967; Myers and Daly 1983) among islands in the Bocas del Toro archipelago (Figure 1). There is also dramatic divergence between some populations on mainland Bocas del Toro, which are typically separated by regions of low swamp uninhabited by *D. pumilio* (Daly and Myers 1967).

In contrast, *Minyobates* sp. and *Phyllobates lugubris*, two closely related species of dart-poison frogs that are sympatric with *D. pumilio* in the Bocas del Toro archipelago, show little variation in color or pattern across their ranges. However, both display the bright, aposematic coloration characteristic of the dart-poison frogs. Furthermore, variation in color and pattern across different *Minyobates* and *Phyllobates* species provides evidence of the evolutionary potential for color and pattern variety in both of these genera (Silverstone 1975, 1976). Owing to common descent (family Dendrobatidae) and shared geography, *Minyobates* sp. and *P. lugubris* serve as natural controls in our analysis of divergence between populations of *D. pumilio* in the Bocas del Toro archipelago.

If populations of all three species of dart-poison frogs have had the potential to drift apart in color and pattern, then a neutral model of evolution would predict a correlated genetic divergence. This correlation should be particularly evident at fourfold degenerate sites, which are considered to be under low levels of selective constraint. Thus the processes of genetic drift should yield similar levels of interpopulational divergence at fourfold degenerate sites if all three species share a similar history. Alternatively, perhaps the extreme phenotypic divergence noted for island populations of *D. pumilio* indicates longer periods of population separation than that experienced by the other two species. In this case we would expect to see greater genetic divergence between geographic populations of *D. pumilio* than would be observed between populations of *P. lugubris* and *Minyobates* sp. We tested our genetic expectations by investigating mitochondrial DNA (mtDNA) sequence divergence between conspecific populations of *D. pumilio*, *P. lugubris*, and *Minyobates* sp. from island and mainland populations in the Bocas del Toro archipelago.

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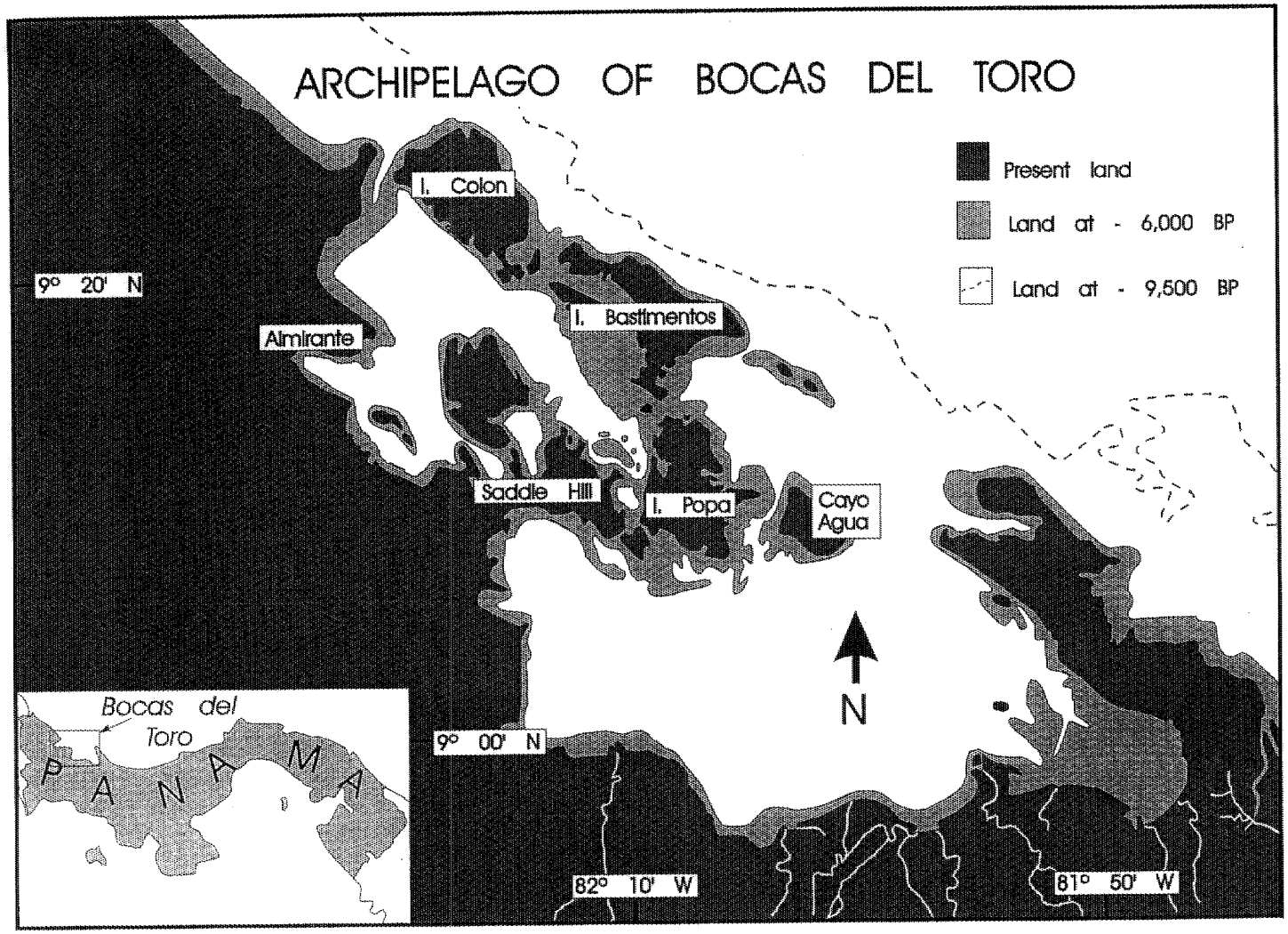

Figure 1. Map of the Bocas del Toro archipelago showing frog collection localities and continental and island margins at three different sea levels.

Materials and Methods

Collections

Figure 1 shows the localities in the Bocas del Toro archipelago where the focal species in this study where collected. Collection localities for outgroup species are not shown. We sequenced 35 dendrobatid frogs representing the following localities and species: Bocas del Toro, Panama (locales mapped in Figure 1): Isla Colon, *D. pumilio* (n = 2) and *Minyobates* sp. (n = 2); Isla Bastimentos (west), *D. pumilio* (n = 2), *P. lugubris* (n = 2); Isla Bastimentos (east), *D. pumilio* (n = 2) and *Minyobates* sp. (n = 2); Isla Popa, *D. pumilio* (n = 2), *P. lugubris* (n = 1) and *Minyobates* sp. (n = 1); Isla Cayo de Agua, *D. pumilio* (n = 1) and *Minyobates* sp. (n = 2); Almirante, *D. pumilio* (n = 1) and *P. lugubris* (n = 2); Saddle Hill, *D. pumilio* (n = 1), *Minyobates* sp. (n = 2), and *P. lugubris* (n = 3). Owing to the scale of the map shown in Figure 2 the following locales are not displayed: San Blas, Panama: *M. minutus* (n = 2) and *Colostethus talamancae* (n = 1); Fortuna, Panama: *D. speciosus* (n = 1); Corcovado, Costa Rica: *P. vitattus* (n = 2); Santo Domingo, Ecuador: *D. histrionicus* (n = 1).

Sample sizes from each locale were low owing to Panamanian (INRENARE) regulations regarding collections of species in the family Dendrobatidae (all are cites listed). Current PCR-based lab procedures permit toe clips to be utilized as a source of DNA. However, at the time these dendrobatids were collected it was not yet realized that sufficient DNA could be extracted from very small tissue samples. Thus whole frogs were preserved in liquid nitrogen and stored at −70°C until analyzed. Tissue dissections on these small frogs were extensive and as a result the carcasses were not maintained as vouchers.

DNA Extractions

Muscle tissue (0.5 g) was homogenized in 300 μl of lysis buffer (100 mM EDTA, 100 mM Tris pH 7.5, 1% SDS). Samples were incubated overnight with 25 μl of proteinase K solution (20 mg proteinase K/ml in 50% glycerol) at 65°C with constant mixing. The homogenate was centrifuged for 3 min at 14,000 rpm. The supernatant was transferred to a new tube and extracted once with equal volumes of equilibrated phenol, once with phenol-chloroform-isoamyl alcohol (25:24:1), and once with chloroform-isoamyl alcohol (24:1). DNA was precipitated overnight at −20°C with ethanol and 2.5 M ammonium acetate (pH 7.5) and centrifuged for 10 min at 14,000 rpm. The resulting pellet was rinsed once with 70% ethanol, vacuum dried, resuspended in 100 μl of dH_20, and stored at −20°C.

PCR Amplifications

Initial polymerase chain reaction amplifications were performed in 50 μl reactions containing 1 μl of genomic DNA, 5 μl of

10× buffer (100 mM Tris pH 8.3, 20 mM $MgCl_2$, 500 mM KCl, 0.1% gelatin), 2.5 μl each of 10 mM stock solutions of the cytochrome b primers (L14817 and H15175; Kocher et al. 1989), 5 μl of dNTP mix (2.0 mM dATP, 2.0 mM dGTP, 2.0 mM dCTP, 2.0 mM dTTP), and 0.25 μl (1.25 units) of *Taq* polymerase. The samples were overlaid with a drop of mineral oil and cycled thirty times on a Perkin-Elmer thermal cycler using the following conditions: 94°C for 45 s (denaturing step), 50°C for 45 s (primer annealing step), and 72°C for 60 s (primer extension step). Following amplification the PCR products were run in 1.5% low melting point agarose gels in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and stained with ethidium bromide. Typically a single band was visualized, and this fragment was cut from the gel and diluted in 400 μl of dH_2O.

Following this procedure, 1 μl of the diluted PCR fragment was used in an asymmetric amplification in which one primer (the limiting primer) was present at one-one hundredth of the original concentration in the first PCR. Two asymmetric amplifications were carried out for each individual, one with L14817 and one with H15175. After amplification the samples were desalted and concentrated over Centricon 30 columns.

DNA Sequencing

Dideoxy sequencing reactions were carried out using the Sequenase 2.0 kit (United States Biochemical Co., Cleveland, Ohio) and following the vendor's protocol. The sequencing reactions were electrophoresed through 6.0% Long Ranger gels (AT Biochemical Inc., Malvern, Pennsylvania) which were run for 2, 4, 7 and 9 h. The gels were dried and exposed to autoradiograph film for 12–48 h. DNA sequences were read from autoradiographs into the MacVector sequence alignment program (IBI-Kodak, New Haven, Connecticut) using the IBI gel reader. Sequence fragments from different gels were aligned with the MacVector sequence alignment algorithm, and consensus sequences were constructed for each individual. Both light and heavy strand sequences were determined, and on average, sequences are based on almost full overlap of light and heavy strand sequence.

Data Analysis

Cladistic analysis was carried out with PAUP, v. 3.1 (Swofford 1993) using maximum parsimony with *C. talamancae* as the designated outgroup. The genus *Colostethus* is considered to be an outgroup to the other (toxic) members of the family Dendrobatidae (Myers 1987; Myers et al. 1991). Starting trees were obtained using a random stepwise addition procedure, and tree bisection-reconnection branch swapping was performed with PAUP's MULPARS option in effect. Because our study focuses on intraspecific variation, we did not differentially weight nucleotide characters in the parsimony analyses presented here. We did, however, use an a posteriori transversion-transition cost matrix of 4:1 and the same tree building procedures described above to check the results obtained with unweighted characters. The consensus tree presented here (Figure 2) was constructed using the 50% majority rule. Decay indices were calculated based on a heuristic search (addition sequence random, search simple, constraints set to all shortest trees where node defining a particular clade is not found) using PAUP and AUTODECAY (Eriksson and Wilkström 1995).

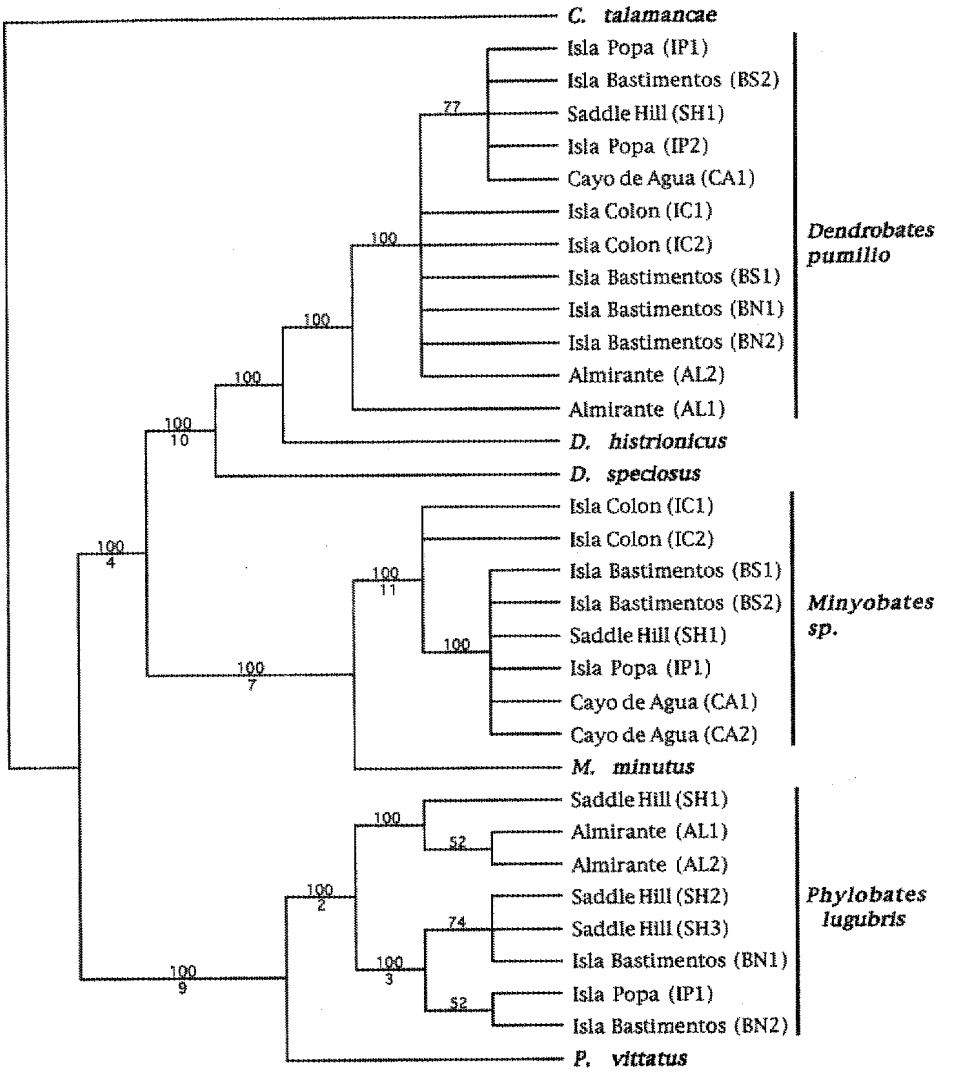

Figure 2. Parsimony 50% majority rule consensus tree of Bocas del Toro dendrobatid frogs and outgroups. Thirty-one trees of score 203 were found with CI = 0.67. Group frequencies are shown above the branches and decay indices are shown beneath the branches.

Distance analysis was performed using MEGA (Kumar et al. 1993). Genetic distances were calculated using Kimura's (1980) two-parameter model and were summarized using the neighbor-joining (NJ) algorithm. Estimates of confidence are based on 1,000 replications of the bootstrap resampling method. Only bootstrap values greater than 85% are shown on the NJ tree (Figure 3). Standard errors of branch lengths and their probabilities were determined using the program METREE (Rzhetsky and Nei 1994).

Sea Level Reconstruction

Figure 1 shows a map of the Bocas del Toro archipelago. Contour lines show sea

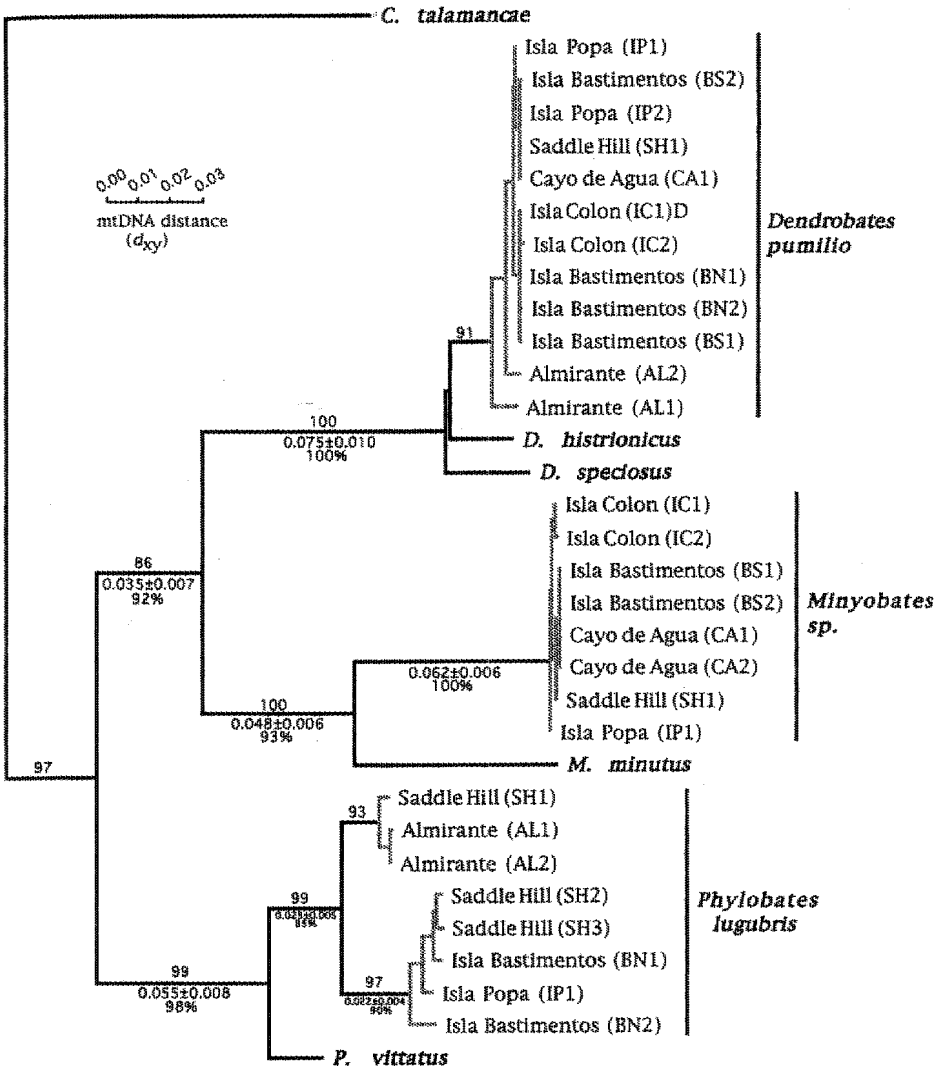

Figure 3. Neighbor-joining tree of Bocas del Toro dendrobatid frogs and outgroups. Bootstrap values greater than 85% are shown above the branches. The lengths of the branches along with their standard errors for the major clades are shown below their respective branches. The probability of these branches being greater than zero is shown below their respective branch lengths.

levels that were reconstructed from paleontological data and geological data by Tony Coates (personal communication) at 9,500 years ago, 6,000 years ago, and at present as applied against bathymetry charts of the Bocas del Toro archipelago. The modern Bocas del Toro archipelago dates to approximately 6,000 years before the present. The cyclical nature of sea level change suggests earlier episodes when the islands of the Bocas del Toro archipelago might have been isolated from one another and from the mainland.

Results

We analyzed mtDNA sequences representing 8 species and 35 individuals of Dendrobatid frogs. Our study focused on *D. pumilio* (n = 12), *P. lugubris* (n = 8), and *Minyobates* sp. (n = 8) collected from seven localities in the Bocas del Toro archipelago. In addition, we analyzed additional species representing each of the three aforementioned genera and *Colostethus*. A total of 292 bp of the mtDNA cytochrome b gene, corresponding to positions 14,843–15,134 in the human mtDNA genome (Anderson et al. 1981), were sequenced for each individual. The DNA sequences for the 33 frogs analyzed in this study have been reported to GenBank (accession numbers U70140-70172).

The nucleotide base composition at first, second, and third positions and fourfold degenerate sites in Dendrobatidae mtDNA cytochrome b segment was generally consistent with the pattern seen in most vertebrates (e.g., Kocher et al. 1989) and demonstrated a characteristic and strongly antiguanine bias at third positions (A1, 24.5; T1, 30.0; C1, 19.6; G1, 25.9) (A2, 17.3; T2, 44.2; C2, 20.6; G2, 18.0) (A3, 22.9; T3, 30.2; C3, 42.8; G3, 4.1) (A4, 23.2; T4, 16.5; C4, 53.6; G4, 6.8). The standard deviation around these mean values was 0.5–2%, except for thymine and cytosine residues at third position and fourfold degenerate sites which had a standard deviation of 5.2–5.8%. The outgroup, *C. talamancae*, deviated considerably from the mean and from all members of the ingroup at these sites. For example, T4 and C4 proportions observed in *C. talamancae* were 28.6 and 31.4, respectively. The nearest values observed in the ingroup were T4 at 22.9 and C4 at 45.7.

Figures 2 and 3 show the phylogenetic relationships of the three study species and their putative sister taxa. Both cladistic and distance analyses demonstrated that the populations from each of the three species form monophyletic groups and, as measured by divergence between mtDNA cytochrome b sequences, were similarly differentiated from their sister taxa. Whether we used the average corrected sequence divergence at all sites or at fourfold degenerate sites, genetic distances between populations of *D. pumilio* (0.005 ± 0.006 and 0.020 ± 0.027, respectively) were intermediate between those among populations of *Minyobates* sp. (0.001 ± 0.002 and 0.007 ± 0.008) and *P. lugubris* (0.030 ± 0.020 and 0.130 ± 0.086). Mitochondrial DNA distances among all populations within each species were low and generally well below the genetic distances observed between congeneric species (Figure 3). Character weighting did not alter our results.

Discussion

Daly and Myers (1967) tentatively placed the different populations of *D. pumilio* (sensu lato) from the Bocas del Toro archipelago into the single species *D. pumilio* Schmidt. Myers and Daly (1976) argued that populations from the western part of the Bocas del Toro archipelago on through Costa Rica form a single species on the basis of similarities in toxin characteristics, karyotypes, and calling parameters, including the number of notes per second and note and call duration. The low degrees of mtDNA divergence among the populations of *D. pumilio* are consistent with that conclusion.

Thus the mtDNA data do not suggest any pronounced difference in population histories that might explain the marked

difference in color and pattern differentiation between the species. Hence, it seems unlikely that time of divergence alone can explain the dramatic difference between these species in the degree of divergence among populations in color and pattern.

Dendrobates, Phyllobates, and *Minyobates* all produce toxic alkaloids. The bright coloration characteristic of many dendrobatids is believed to be aposematic in function, probably serving to warn visually oriented predators that have color vision, such as birds (Daly and Myers 1967). The *D. pumilio* populations differ from one another in the number and type of toxins they produce, but toxin characteristics do not appear to be correlated with the brightness of color found in particular populations (Daly and Myers 1967; Myers and Daly 1983). The extreme island-to-island variation in coloration and pattern found in *D. pumilio* populations is puzzling given its presumed aposematic function, because natural selection should favor individuals that closely resemble other toxic frogs exposed to the same individual predators, whether they are from the same species or not (Mullerian mimicry; Muller 1879).

The lack of correlation between toxicity and coloration may result from dietary differences across dendrobatid frogs. Recent research indicates that at least some dendrobatid toxins are accumulated through dietary uptake (Daly et al. 1994a,b). However, this does not explain why *D. pumilio* populations have evolved marked differentiation, while *P. lugubris* and *Minyobates* sp. populations have not.

Color and pattern divergence in *D. pumilio* might have arisen as a result of selection in differing ecological regimes. However, ecological differences seem unlikely to explain the difference between *D. pumilio* and the other two species in the amount of color and pattern divergence. All three species are diurnal, active frogs that live on the forest floor and are frequently found within a few centimeters of each other. In each of the populations sampled in this study, either *Minyobates* sp. or *P. lugubris,* or both, were collected in the same area as *D. pumilio.*

Given these ecological similarities, what selection pressure could have driven interpopulation divergence in color and pattern in *D. pumilio* while not affecting either *P. lugubris* or *Minyobates* sp.? One possible explanation is differences among the species in parental investment and sexual selection. In *D. pumilio* and closely related species, females carry tadpoles to small pools of water and return periodically to lay trophic eggs which the tadpoles require for survival (Brust 1993; Weygoldt 1980). Males in *D. pumilio* provide relatively little parental care compared to females; males do not carry or feed tadpoles, but may periodically attend clutches for brief periods, shedding water on them (Weygoldt 1980, 1987). *P. lugubris,* like other members of its genus, has uniparental male care (Weygoldt 1987; Zimmermann and Zimmermann 1988). Recent field research by K. Summers on *M. minutus,* a closely related sister species of *Minyobates* sp., revealed that parental care is performed by the male.

During a 5 month field study of *M. minutus* in San Blas, Panama, marked males were observed carrying tadpoles to pools of water in bromeliads, whereas marked females were not. Eight frogs that were collected outside the study area while carrying tadpoles were sexed by dissection; all were males (Summers K, unpublished data). Male parental care has also been found in more distantly related species of *Minyobates* (Myers and Daly 1980), suggesting that male parental care is characteristic of this genus.

Sexual selection theory (Trivers 1972; Williams 1966) predicts that female choice for male ornamentation will be particularly strong in species in which females provide more investment in offspring than do males. In species with substantial male parental care, female choice is constrained because male parental care exerts direct selection on female preferences (Kirkpatrick 1985) and rejection is more costly to females (Trivers 1972). In species where males provide little or no parental investment, these constraints are relaxed. In turn, rapid divergence in sexually selected traits can occur because females may prefer different forms of the trait in different populations (Darwin 1871; West-Eberhard 1983).

D. pumilio are diurnal and have a long courtship (Limerick 1980), which may allow females to assess male color and pattern. Most frogs are believed to have dichromatic vision (Donner and Reuter 1976), and there is evidence that some species, including *D. pumilio,* have color discrimination capabilities (Hailman and Jaeger 1974).

One potential problem with this explanation is that most sexually selected characteristics are sexually dimorphic (Trivers 1972), whereas there is no obvious dimorphism in color or pattern between male and female *D. pumilio.* However, there are two possible explanations for a lack of pronounced dimorphism in *D. pumilio.* First, it is possible that female coloration is genetically correlated with that of males, causing selection on male coloration to produce a correlated response in female coloration (Irwin 1994). Second, because bright coloration and contrasting patterns probably serve as warnings to predators in *D. pumilio* (Daly and Myers 1967), selection within populations may favor females with the same color and pattern as males.

Sexual selection associated with female parental care may have caused rapid divergence in coloration and rapid speciation in cichlid species flocks in Lake Victoria and Lake Malawi in East Africa (Meyer 1993). Species from these flocks are similar to the divergent populations of *D. pumilio* in that they show low degrees of genetic divergence, they inhabit ecologically similar or identical environments, and they differ primarily in color and pattern (Dominey 1984).

The molecular phylogenetic and biogeographic evidence (Figures 1–3) suggests that divergence in color and pattern has been rapid among *D. pumilio* populations. It is possible that these populations have only been evolving in isolation for 6,000 years or less (Figure 1). Female choice can account for this rapid differentiation and would not be expected to play a role in *P. lugubris* or *Minyobates* sp. populations. In these species female choice is likely to be constrained by male parental care (Trivers 1972; Williams 1966). Our observations are consistent with the hypothesis that sexual selection drove the rapid divergence in color and pattern between populations of *D. pumilio.*

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