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Geographic and Seasonal Variation in Alkaloid-Based Chemical Defenses of *Dendrobates pumilio* from Bocas del Toro, Panama

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Abstract Poison frogs contain an alkaloid-based chemical defense that is derived from a diet of certain alkaloid-containing arthropods, which include mites, ants, beetles, and millipedes. Variation in population-level alkaloid profiles among species has been documented, and more than 800 different alkaloids have been identified. In the present study, we examine individual alkaloid variation in the dendrobatid poison frog *Dendrobates pumilio* among seven populations and between two seasons on Isla Bastimentos, located in the Bocas del Toro archipelago of Panama. Alkaloid profiles vary among populations and between seasons, illustrating that chemical defense in this species can vary on a small spatial and temporal scale. Alkaloid variation among populations is marginally correlated with geographic distance, and close populations have profiles more similar to each other than to distant populations. Individuals within populations also vary in alkaloid profiles. Differences are attributed to both spatial and temporal variations in the availability of alkaloid-containing arthropods. Many of the alkaloids present in the skin of *D. pumilio* appear likely to be of ant origin, supporting the importance of myrmecophagy in chemical defense among poison frogs. However, a variety of frog skin alkaloids was recently detected in mites, suggesting that mites may also play an important role in chemical defense.

Keywords Poison frogs · Dendrobatid · *Dendrobates pumilio* · Alkaloids · Arthropods · Ants · Mites · Chemical defense

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Introduction

Chemical defense in animals generally involves the manufacture or acquisition of defensive compounds from other organisms (Mebs, 2001). Although manufacturing defensive compounds is more common among animals, their acquisition from other organisms has convergently evolved in numerous lineages (Termonia et al., 2001). Animals commonly acquire defensive compounds from other organisms through the uptake, accumulation, in some cases modification, and storage of compounds (or precursors) that were originally present in other organisms, including microorganisms, plants, or other animals (Mebs, 2001; Termonia et al., 2001). Animals that employ this type of defensive strategy are dependent on symbiotic relationships with organisms capable of manufacturing defensive chemicals or a particular diet of chemically defended prey (Mebs, 2001).

Arthropods represent the largest group of animals known to acquire defensive compounds through diet, and this ability is known for lepidopterans, orthopterans, hemipterans, coleopterans, and hymenopterans (Boppre, 1990; Hartmann and Witte, 1995; Hartmann and Ober, 2000; Klitzke and Trigo, 2000). In addition to arthropods, flatworms (Kubanek et al., 1995), nudibranch mollusks (Cimino and Ghiselin, 1998; Fahey and Garson, 2002), birds (Dumbacher et al., 2000, 2004), amphibians (Daly et al., 1994a,b, 2000, 2002, 2005; Jones et al., 1999; Saporito et al., 2003, 2004; Clark et al., 2005), and possibly snakes (Mori and Burghardt, 2000) have also been reported to acquire defensive compounds from dietary sources.

Poison frogs represent a group of organisms that are chemically defended from predation and possibly microorganisms by the dietary uptake of lipophilic alkaloids from arthropod prey (Daly and Myers, 1967; Daly et al., 1994a,b, 2000, 2002; Jones et al., 1999; Saporito et al., 2003, 2004; Clark et al., 2005; Macfoy et al., 2005). More than 800 alkaloids, representing at least 24 different structural classes, have been isolated from the skin of brightly colored poison frogs from four different anuran families (Dendrobatidae from Central and South America; Bufonidae from South America; Mantellidae from Madagascar; Myobatrachidae from Australia; Daly et al., 2005). The ability to uptake certain alkaloids through diet has been experimentally demonstrated in dendrobatid frogs of the genera *Dendrobates*, *Phyllobates*, and *Epipedobates* (Daly et al., 1994b), and mantellid frogs of the genus *Mantella* (Daly et al., 1997). It seems likely that bufonid toads of the genus *Melanophryniscus* will share a similar ability to uptake alkaloids (Garraffo et al., 1993a). Myobatrachid frogs of the genus *Pseudophryne* appear to biosynthesize pseudophrynamine alkaloids, yet are able to accumulate dietary provided pumiliotoxin alkaloids (Smith et al., 2002). Frogs of the genus *Dendrobates* have been shown to efficiently convert a dietary pumiliotoxin alkaloid (**251D**) to a more toxic allopumiliotoxin alkaloid (**267A**; Daly et al., 2003), and one species of the genus *Pseudophryne* appears to be able to metabolize a dietary pumiliotoxin (**307A**) by both reduction and hydroxylation (Smith et al., 2002), representing the only known incidents of alkaloid modification in poison frogs.

A variety of alkaloids are present in arthropods (for a review see, Jones and Blum, 1983; Numata and Ibuka, 1987; Braekman et al., 1998 and references within), and the ability of poison frogs to accumulate alkaloids through diet suggests that sequestration of these compounds from arthropod prey accounts for their presence in the skin of these frogs (with the exception of the pseudophrynamines of

myobatrachid poison frogs). Several alkaloid classes found in the skin of poison frogs have been identified in arthropods such as oribatid mites (Takada et al., 2005; Saporito et al., unpublished data), myrmicine, formicine, and ponerine ants (Daly et al., 1994a, 2000; Jones et al., 1999; Saporito et al., 2004; Clark et al., 2005), siphonotid millipedes (Saporito et al., 2003; Clark et al., 2005), and coccinellid and melyrid beetles (Ayer and Browne, 1977; Dumbacher et al., 2004). These arthropods represent the likely dietary sources for certain alkaloids found in poison frogs. Therefore, their presence in poison frogs is the result of uptake, accumulation, in certain cases modification, and storage of alkaloids from a diet of alkaloid-containing arthropods.

Alkaloid profiles (a measure of the number, type, and amount of alkaloids) are known to vary spatially and temporally among and within species of poison frogs (Myers and Daly, 1976, 1980; Daly et al., 1987, 1992, 1994a,b, 1996, 2002; Garraffo et al., 1993a,b; Myers et al., 1995; Clark et al., 2005; Mebs et al., 2005; and references within). In most cases, alkaloid variation has been described on a population level, in which samples of frogs collected from a certain population are pooled for chemical analysis (examples include Daly et al., 1987, 1996, 2002; Garraffo et al., 1993a,b; Mortari et al., 2004; and references within). Alkaloid variation among individuals within a population has been reported, but only in a few instances (see Daly et al., 1992, 1994a,b; Clark et al., 2005; Mebs et al., 2005; Myers et al., 1995). Although it is generally assumed that alkaloid variation within populations is less than variation among populations (see Daly et al., 1992), relatively few studies have directly addressed this issue. Changes over time in profiles have also been documented (Daly et al., 1987, 2002; Myers et al., 1995); however, no studies have specifically examined the question.

This study investigated the degree of geographic and temporal variations in alkaloid chemical defense within and among populations of the dendrobatid poison frog, *Dendrobates pumilio*, on Isla Bastimentos, located within the Bocas del Toro archipelago of Panama. The natural geographic range of *D. pumilio* extends from lowland Caribbean rainforests of southern Nicaragua through Costa Rica and into northwestern portions of Panama (Myers and Daly, 1983). Population-level alkaloid profiles have been shown to vary among populations of *D. pumilio* in the northwestern portions of Panama within and among islands in the Bocas del Toro archipelago, on the mainland bordering the archipelago, and in a few cases over time (Daly et al., 1987, 2002, unpublished data). In this study, we take a multivariate statistical approach to examine differences in individual alkaloid profiles within and among populations of *D. pumilio* over a small geographic range and between two different seasons.

Methods and Materials

Frog Collections

A total of 70 *D. pumilio* were collected from seven different populations located on Isla Bastimentos, Bocas del Toro Province, Panama (Fig. 1 and Table 1). Five *D. pumilio* were collected at each of the seven populations during the dry and wet seasons of 2003 (February 2–8 and August 20–23, respectively), for a total of 10

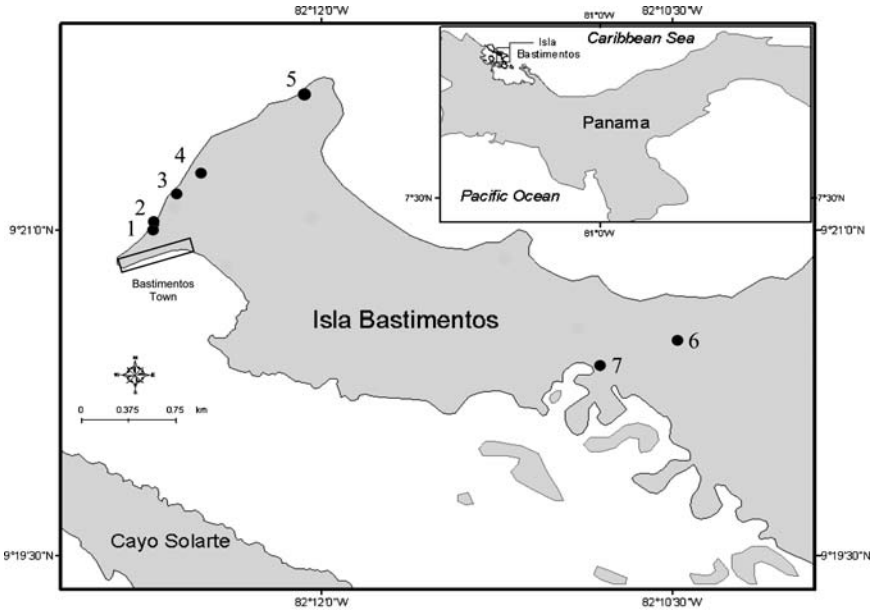


Fig. 1 Map of research sites on Isla Bastimentos, Bocas del Toro, Panama

frogs per population. At each of the seven sites, one 45 × 45 m plot was established. Frogs were captured by hand and the same plots were used in both seasons. Individuals were measured snout to vent (SVL) to the nearest 0.5 mm, and sex was determined. Only adult frogs were used (>20 mm SVL). All frogs were euthanized in the field laboratory, and individual *D. pumilio* skins were stored in uniquely marked 1.7-ml plastic vials containing 100% methanol at ambient temperature. All

Table 1 *Dendrobates pumilio* collection sites and descriptions on Isla Bastimentos, Bocas del Toro, Panama

Site	Name	GPS coordinates	Site description
1	Brust	9°20.996′N, 82°12.726′W	Numerous coconut palms; leaf-litter abundant
2	Cyclanthus slope	9°20.364′N, 82°10.807′W	Numerous *Cyclanthus* sp.; leaf-litter abundant
3	Tall Heliconia	9°21.169′N, 82°12.627′W	Numerous *Heliconia* sp.; leaf-litter sparse
4	Big Rock Forest	9°21.250′N, 82°12.519′W	Numerous *Cyclanthus* sp. and cacao; leaf-litter abundant
5	Faro	9°21.618′N, 82°12.074′W	Secondary forest; leaf-litter abundant
6	Red Frog Beach Hill	9°20.490′N, 82°10.486′W	Secondary forest; leaf-litter abundant
7	Shortcut	9°21.021′N, 82°12.704′W	Secondary forest; leaf-litter abundant

voucher specimens were deposited in the herpetological collection at Florida International University.

Alkaloid Extraction and Isolation

Individual alkaloid fractions were generated for all 70 *D. pumilio*; however, the protocol for preparing the individual fractions differed between seasons. Alkaloid fractions for the dry season samples was prepared as described by Daly et al. (1994b), whereas alkaloid fractions for the wet season samples were obtained from a modified version of this protocol (see below). The modified version was used to reduce analysis time.

Dry Season Samples

Each skin was cut into small pieces and macerated (with a mortar and pestle) × 3, each time with 5 ml methanol. The combined methanol extract was diluted with 15 ml water and extracted × 3, each time with 15 ml chloroform. Combined chloroform layers were concentrated to a small volume (~5 ml) *in vacuo* at 35°C with a rotary evaporator. Following concentration, 20 ml *n*-hexane were added to the concentrated chloroform layer. This solution was extracted × 3, each time with 15 ml of 0.1 N HCl. The combined 0.1 N HCl fractions were adjusted to pH 9.0 with 2 N aqueous ammonia, followed by extraction × 3, each time with 15 ml chloroform. The combined chloroform extract was dried with anhydrous Na_2SO_4 and evaporated to dryness at 35°C *in vacuo* with a rotary evaporator. The resulting alkaloid residue was dissolved in sufficient methanol so that 100 μl of this alkaloid fraction corresponded to 100 mg of the original wet weight of the skin. In the dry season samples, it appeared that the recovery of alkaloids was less than expected, although there was no apparent explanation for the poor yield.

Wet Season Samples

Each skin was cut into small pieces and macerated (with a mortar and pestle) × 2, each time with 4 ml methanol. The combined methanol extract was immediately mixed with 8 ml of 0.1 N HCl and extracted × 5, each time with 20 ml hexane. The pH was adjusted to 9.0 with 2 N aqueous ammonia, followed by extraction × 3 with 15 ml chloroform. This solution was dried with anhydrous Na_2SO_4 and evaporated to dryness at 35°C *in vacuo* with a rotary evaporator. Following the original protocol, the resulting alkaloid residue was dissolved in sufficient methanol so that 100 μl of this alkaloid fraction corresponded to 100 mg of the original wet weight of the skin.

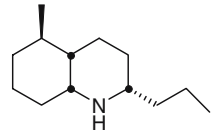
Alkaloid Identification (GC-MS)

All alkaloids within each fraction were identified by using gas chromatography in conjunction with mass spectrometry (GC-MS). Identification of individual alkaloids was based on comparison of retention times and mass spectral data with expected data for known anuran alkaloids. Anuran alkaloids have been assigned a code name, consisting of a bold-faced number corresponding to the nominal mass and a bold-faced letter for identification of individual alkaloids with the same nominal mass

(Daly et al., 1999). A revised, updated tabulation with code names for over 800 anuran alkaloids is now available (Daly et al., 2005).

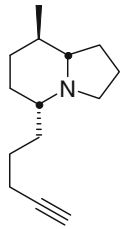
GC-MS analysis for all dry season samples was performed on a Finnigan GCQ instrument with a 25 m × 0.25 mm i.d. Rtx-5 amine fused-silica column (Restek). GC-MS analysis for all wet season samples was performed on the same Finnigan GCQ instrument, however with a 30 m × 0.25 mm i.d. DB-1 fused-silica column (J&W Scientific). GC separation of alkaloids was achieved by using a temperature program from 100 to 280°C at a rate of 10°C per min with helium as the carrier gas. Each extract was analyzed with both electron impact-mass spectrometry (EI-MS) and chemical ionization-mass spectrometry (CI-MS) with ammonia as the reagent

2,5-Disubstituted decahydroquinoline (DHQ):

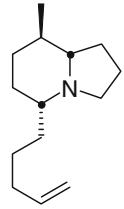


cis-**195A**

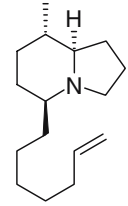
5,8-Disubstitued indolizidines (5,8-I):



205A

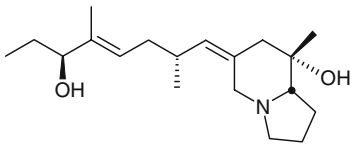


207A

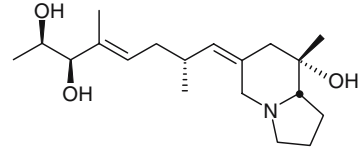


235B''

Pumiliotoxins (PTX):



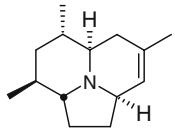
307A



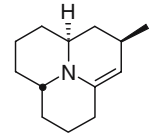
323A

Fig. 2 Structures of the most abundant alkaloids found in *Dendrobates pumilio* on Isla Bastimentos, Bocas del Toro, Panama

Tricyclics (TRI):

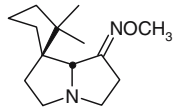


205B

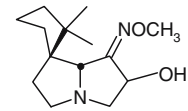


191B

Spiropyrrolizidine oximes (Spiro):

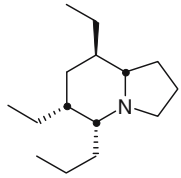


236



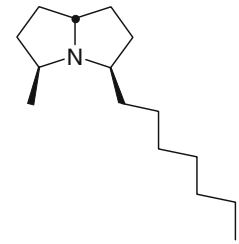
252A

5,6,8-Trisubstituted indolizidine (5,6,8-I):



223A

3,5-Disubstitued pyrrolizidine (3,5-P):



223H

Fig. 2 (continued)

gas. An injection volume of 2 μl was used for each frog alkaloid fraction, corresponding to 2 mg wet weight frog skin.

Alkaloid Quantification (GC-FID)

All alkaloids within each fraction were assessed quantitatively by using gas chromatography in combination with a flame-ionization detector (GC-FID). GC was performed on a Hewlett Packard 5890 with a 6-ft 1.5% OV-1 packed column (2 mm i.d.). GC separation of alkaloids was achieved by using a temperature program from 150 to 280°C at 10°C per min with helium as the carrier gas. An injection volume of 2 μl was used for each alkaloid fraction, corresponding to 2 mg wet weight frog skin.

In order to quantitatively assess the amount of alkaloids within individual frogs, a calibration curve for GC-FID was constructed by using a standard of the alkaloid, decahydroquinoline (DHQ) **195A**. Decahydroquinoline **195A** is the most common alkaloid found in all frog populations from the northwest coast of Isla Bastimentos. Each alkaloid within an individual frog was assigned to one of the following quantitative categories: (1) major alkaloid (present in an amount greater than or equal to 50 μg per 100 mg frog skin), (2) minor alkaloid (present in an amount between 5 and 50 μg per 100 mg frog skin), or (3) trace alkaloid (present in an amount less than 5 μg per 100 mg frog skin). Using this quantification scheme allows for the direct comparison of data from this study to those of previous and current studies conducted on other lipophilic alkaloid-containing frogs and toads.

Statistical Analyses

Variation in individual alkaloid profiles within and among populations of *D. pumilio* were graphically visualized for both seasons by using nonmetric multidimensional scaling (nMDS). Statistical differences in individual alkaloid profiles among populations for each season were detected with a one-way analysis of similarity (ANOSIM). Statistical differences in individual alkaloid profiles among the same populations between seasons were examined with a two-way ANOSIM. Both nMDS plots and ANOSIM results are based on Bray–Curtis dissimilarity matrices. A Mantel test was performed to examine the relationship between geographic distance and alkaloid profile variation among populations for the wet season data. All statistical analyses were performed with the software program PRIMER (version 5) and the PopTools add-in function in Microsoft Excel (written by G. Hood; available at http://www.cse.csiro.au/poptools).

Results

Alkaloids Present in *D. pumilio*

A total of 153 alkaloids, representing 16 different structural classes, were identified from *D. pumilio* skin extracts. Individual frogs contained an average of 17 different alkaloids (range 6–31 alkaloids). Populations also contained an average of 17

Table 2 The most abundant alkaloids present in each population of *D. pumilio* on Isla Bastimentos, Panama

Site	DHQ	3,5-P	5,8-I			5,6,8-I	Tricyclic		Spiro		PTX	
	195A	**223H**	**205A**	**207A**	**235B**	**223A**	**205B**	**191B**	**236**	**252A**	**307A**	**323A**
1	+				+		+	+	+	+	+	+
2	+		+	+	+		+	+	+	+	+	+
3	+	+			+	+	+	+			+	+
4	+	+			+		+				+	+
5	+		+	+		+						
6	+		+	+								
7	+		+	+	+	+						

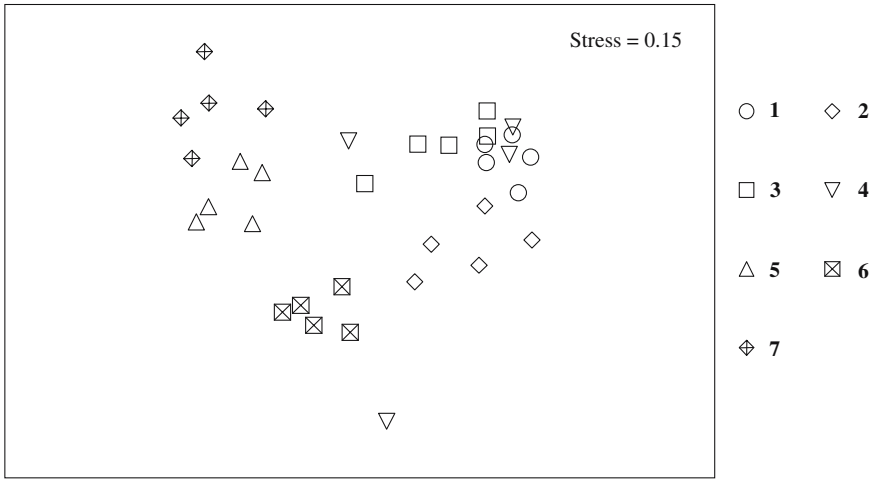


Fig. 3 Nonmetric multidimensional scaling (nMDS) plot of *D. pumilio* alkaloid profiles among seven populations on Isla Bastimentos during the dry season. nMDS plot is based on the presence/absence of alkaloids. Each symbol represents an individual frog from a specific population. Site numbers correspond to map in Fig. 1

different alkaloids (range 9–31 alkaloids). Decahydroquinoline **195A** was found in all frog skin extracts from each of the seven sites during both seasons, and represents the most common and widespread alkaloid among populations of *D. pumilio* on Isla Bastimentos. Decahydroquinoline **195A** is thought to be obtained from myrmicine ants that are found microsympatrically with dendrobatid frogs containing this alkaloid (Daly et al., 2000, 2002). Based on the presence of an

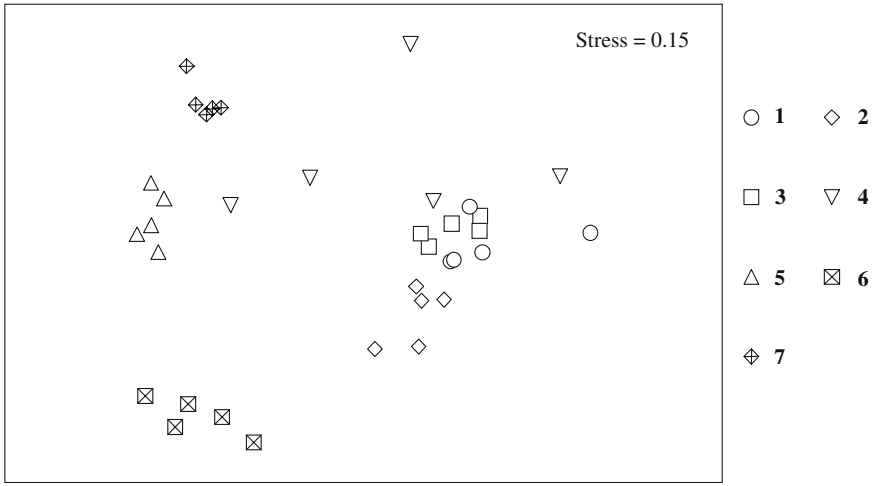
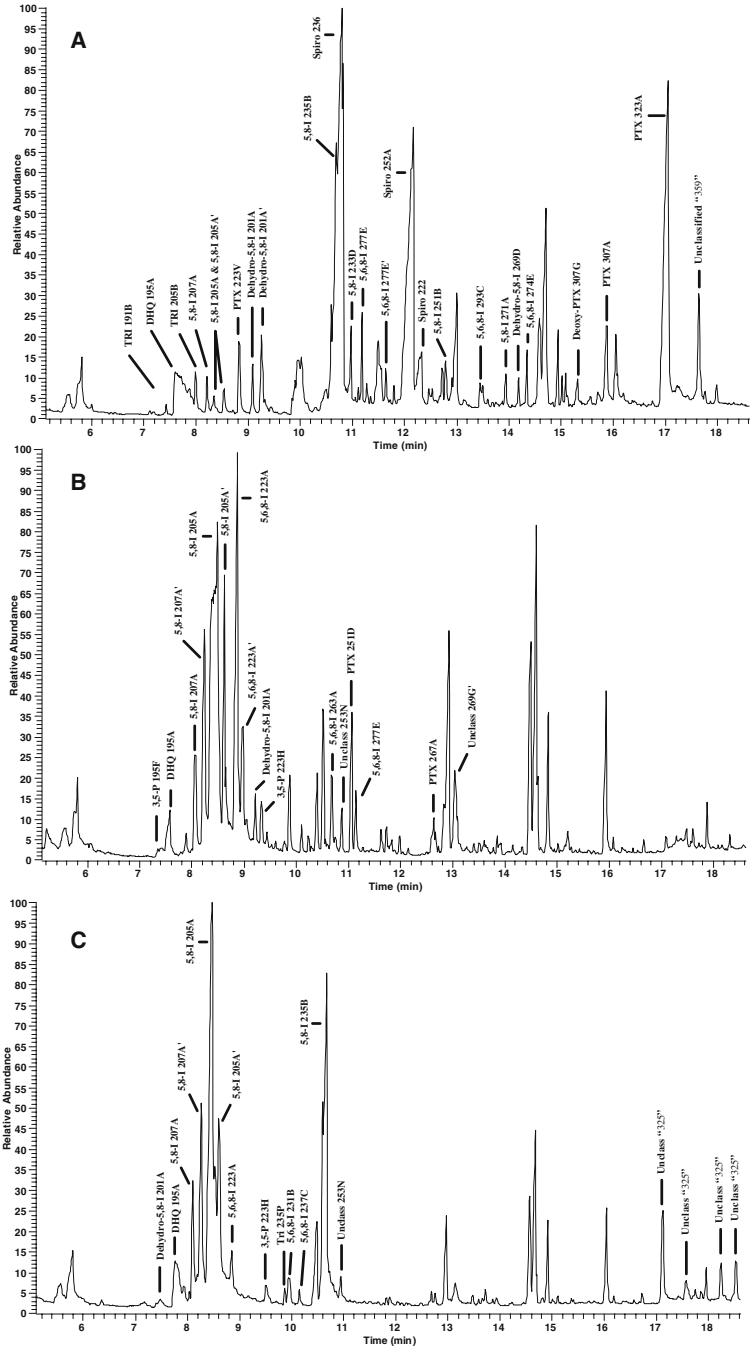


Fig. 4 Nonmetric multidimensional scaling (nMDS) plot of *D. pumilio* alkaloid profiles among seven populations on Isla Bastimentos during the wet season. nMDS plot is based on major, minor, and trace alkaloids. Each symbol represents an individual frog from a specific population. Site numbers correspond to map in Fig. 1


A
Relative Abundance
Time (min)
TRI 191B
DHQ 195A
TRI 205B
5,8-I 207A
5,8-I 205A & 5,8-I 205A'
PTX 223Y
Dehydro-5,8-I 201A
Dehydro-5,8-I 201A'
5,8-I 235B
Spiro 236
5,8-I 233D
5,6,8-I 277E
5,6,8-I 277E'
Spiro 252A
Spiro 222
5,8-I 251B
5,6,8-I 293C
5,8-I 271A
Dehydro-5,8-I 269D
5,8-I 274E
Deoxy-PTX 307G
PTX 307A
PTX 323A
Unclassified "359"
B
Relative Abundance
Time (min)
3,5-P 195F
DHQ 195A
5,8-I 207A
5,8-I 207A'
5,8-I 205A
5,8-I 205A'
5,6,8-I 223A'
Dehydro-5,8-I 201A
3,5-P 223H
5,6,8-I 263A
Unclass 253N
PTX 251D
5,6,8-I 277E
5,6,8-I 223A
PTX 267A
Unclass 269G'
C
Relative Abundance
Time (min)
Dehydro-5,8-I 201A
DHQ 195A
5,8-I 207A
5,8-I 207A'
5,8-I 205A
5,8-I 205A'
5,6,8-I 223A
3,5-P 223H
Tri 235P
5,6,8-I 231D
5,6,8-I 237C
5,8-I 235B
Unclass 253N
Unclass "325"
Unclass "325"
Unclass "325"
Unclass "325"

alkaloid in at least three out of the five frogs sampled from each site (excluding alkaloids present in trace amounts), the other most abundant alkaloids in populations of *D. pumilio* were 5,8-disubstituted indolizidines (5,8-Is) **205A**, **207A**, and **235B′**, pumiliotoxins (PTXs) **307A** and **323A**, tricyclics (TRIs) **205B** and **191B** (propyleine), spiropyrrolizidines (Spiros) **236** and **252A**, a 5,6,8-trisubstituted indolizidine (5,6,8-I) **223A**, a 3,5-disubstituted pyrrolizidine (3,5-P) **223H**, and two unclassified alkaloids of apparent molecular weight "321" and "325." Chemical structures for these are shown in Fig. 2 (structures have not yet been proposed for the two unclassified alkaloids).

The 5,8-Is **205A** and **207A** were detected as major or minor alkaloids in most frog skin extracts from sites 2, 5, 6, and 7, whereas the 5,8-I **235B′** was a major or minor alkaloid in most frog skin extracts from sites 1, 2, 3, 4, and 7. The PTXs **307A** and **323A** were detected as major or minor alkaloids in frog skin extracts from sites 1, 2, 3, and 4. The tricyclic **205B** was a major or minor alkaloid in most frogs from sites 1, 2, 3, and 4, whereas the tricyclic **191B** was a major or minor alkaloid in most frogs from sites 1, 2, and 3. The Spiros **236** and **252A** were the major or minor alkaloids in most frogs from sites 1 and 2. The 5,6,8-I **223A** was a major or minor alkaloid in most frogs from sites 3, 5, and 7. The 3,5-P **223H** was a major or minor alkaloid in most frogs from sites 3 and 4. Two unidentified alkaloids, "321" and "325," were detected as major alkaloids in all frogs from site 7, but only during the wet season. Site locations and descriptions are in Table 1 and Fig. 1. The above information is summarized in Table 2.

Alkaloid Variation—Dry Season

Alkaloid profiles for individual frogs during the dry season were significantly different among the seven sites (Global $R = 0.77$, $P < 0.001$). Pairwise comparisons of the seven sites showed no statistical difference in alkaloid profiles between frogs from sites 3 and 4 (Global $R = 0.19$, $P < 0.063$). Differences within and among sites are graphically displayed with nMDS (Fig. 3). Due to low recoveries, profiles for individual frogs during the dry season were statistically analyzed based on untransformed presence/absence of alkaloids.

Alkaloid Variation—Wet Season

Alkaloid profiles for individual frogs during the wet season were significantly different among all seven sites (Global $R = 0.83$, $P < 0.001$). Differences within and among sites are graphically displayed with nMDS (Fig. 4). Profiles for individual frogs during the wet season were statistically analyzed based on untransformed categorical (i.e., major, minor, or trace) alkaloid data. Representative alkaloid profiles for an individual frog from sites 2, 5, and 6 are presented in Fig. 5. Alkaloid

Fig. 5 Gas chromatographs of alkaloid profiles of individual *D. pumilio* from three different populations on Isla Bastimentos, illustrating variation in alkaloids among populations. Only major and minor alkaloids are identified in the chromatographs. Peaks that are not identified represent trace alkaloids or nonalkaloid compounds (i.e., plasticizers, fatty acid methyl esters, etc. that were not removed during the fractionation process). (A) Individual *D. pumilio* from site 2, (B) individual *D. pumilio* from site 5, (C) individual *D. pumilio* from site 6. Site numbers correspond to map in Fig. 1

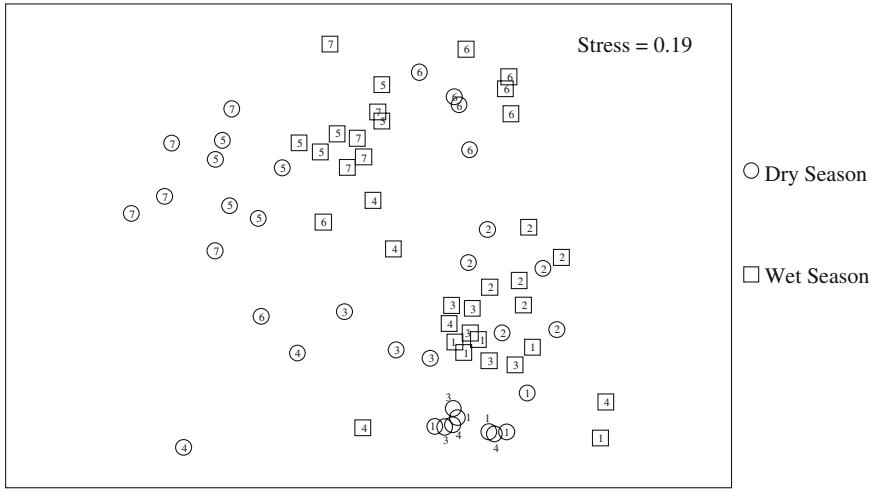


Fig. 6 Nonmetric multidimensional scaling (nMDS) plot of *D. pumilio* alkaloid profiles for individual frogs in seven populations on Isla Bastimentos between the dry and wet season. nMDS plot is based on the presence/absence of alkaloids. Each symbol represents an individual frog from a specific population and season. Site numbers correspond to map in Fig. 1

profiles were positively correlated with geographic distance among all seven sites ($R = 0.51$, $P < 0.001$).

Alkaloid Variation—Dry *vs.* Wet Season

Alkaloid profiles for individual frogs among the seven sites between the dry and wet season were significantly different (Global $R = 0.502$, $P < 0.001$). Differences in alkaloid profiles among sites and between seasons are graphically displayed with nMDS (Fig. 6). Due to low recoveries in the dry season samples, alkaloid profiles for individual frogs among sites and during both seasons were statistically analyzed based on untransformed presence/absence of alkaloids.

Discussion

Alkaloid profiles of *D. pumilio* examined in this study varied significantly among populations and between seasons on Isla Bastimentos, Bocas del Toro, Panama. The seven populations are located in the northwestern region of Isla Bastimentos and no two populations are more than 3.5 km in distance from each other (with most populations being less than 1 km apart; see Fig. 1), demonstrating that chemical defense can vary considerably over a relatively small geographic range. Our results are consistent with a previous study (Daly et al., 2002), which showed that population-level alkaloid profiles varied among five of the same populations. Alkaloid profiles in this study also varied between populations sampled during the dry and wet season, illustrating that chemical defense in *D. pumilio* can vary across short periods of time. Although differences in alkaloid profiles have been documented among

years in certain populations of *D. pumilio* (Daly et al., 1987, unpublished data), changes have never been described for a time period as brief as two seasons.

Variation in alkaloid profiles among populations of *D. pumilio* within a season is related to geographic proximity, and close populations tend to have more similar profiles than distant ones. Myers and Daly (1976) have shown that geographically close populations of *D. histrionicus* share more alkaloids than do distant populations. Myers et al. (1995) reported that sympatric populations of *D. pumilio* and *D. granuliferus* contain more alkaloids in common with each other than with geographically distant populations of *D. granuliferus*. Daly et al. (1992) illustrated that introduced populations of *D. auratus* in Hawaii have alkaloid profiles more similar to each other than those of their ancestral population from Panama. In our study, geographic distance accounted for 51% of the difference in alkaloid profiles among populations of *D. pumilio*, suggesting that the distribution of certain alkaloid-containing arthropods in this region is also related to geographic distance.

Variation in profiles among individuals within populations is less than variation among populations. Daly et al. (1992) stated that there is variation in the amounts of alkaloids found in individuals within populations of *D. auratus*, and suggested that these differences were less than differences among populations. Daly et al. (1994a,b) illustrated that although alkaloid compositions are similar within populations of *D. auratus*, the relative amounts of a given alkaloid are variable. Our study with *D. pumilio* supports these findings. Myers et al. (1995) described individual alkaloid variation within two populations of *D. granuliferus* and one population of *D. pumilio* from Costa Rica, and concluded that there were differences among individual frogs. Clark et al. (2005) recently reported that individual alkaloid profiles varied within populations for three species of *Mantella* from Madagascar. Mebs et al. (2005) recently reported variable levels of alkaloids (particularly the pumiliotoxin **251D**) in individuals of *Melanophryniscus montevidensis* from Uruguay. In our study, individual frogs within a population differed in overall alkaloid profiles (differences in the number of alkaloids present in individual frogs are illustrated in Fig. 7). In some cases, alkaloids that were predominant in one frog were completely absent in another frog from the same population. Clark et al. (2005) suggested that individual variation within populations of *Mantella* represents the possibility that some alkaloid-containing arthropods are rare. Based on the degree of individual alkaloid variation observed here, we agree that certain alkaloid-containing arthropods are rare, and further suggest that in some cases, the distribution of arthropods is confined to small areas and may be the result of localized hatches or migrations (Daly et al., 2002). Differences in alkaloid profiles among *D. pumilio* individuals within a population likely represent local small-scale geographic differences in the availability of certain alkaloid-containing arthropods.

The presence of an alkaloid-based chemical defense in poison frogs (including *D. pumilio*) is a result of the dietary accumulation of alkaloids from a variety of arthropod prey items. Coccinellid beetles contain coccinelline alkaloids as well as some of the structurally related tricyclic alkaloids (Ayer and Browne, 1977), and an oribatid mite contains two coccinelline alkaloids (Takada et al., 2005). Batrachotoxin alkaloids have been identified in melyrid beetles (Dumbacher et al., 2004) and spiropyrrolizidine alkaloids in siphonotid millipedes (Saporito et al., 2003; Clark et al., 2005). Ants appear to be the largest presumed source of alkaloids in poison frogs. In particular, 2,5-disubstituted pyrrolidines, 2,6-disubstituted piperidines, 3,5-disubstituted pyrrolizidines, 3,5-disubstituted indolizidines, 4,6-disubstituted quinoli-

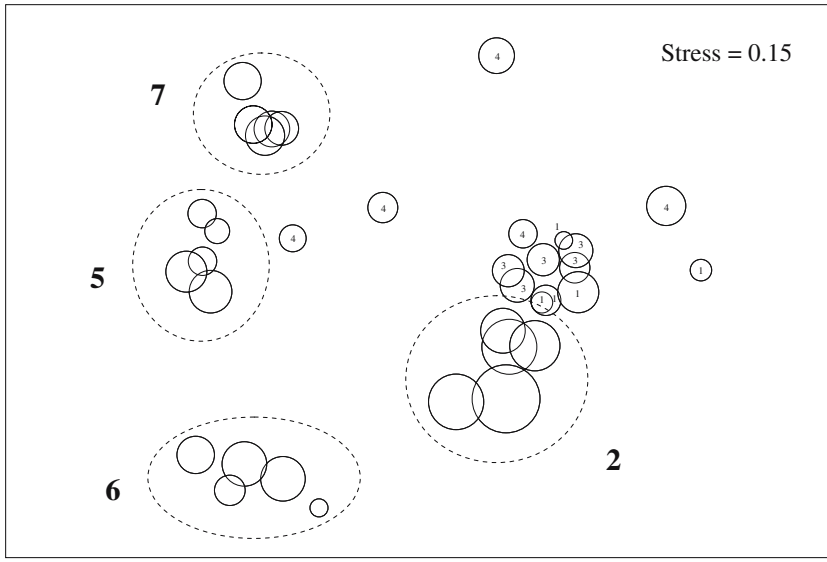


Fig. 7 Nonmetric multidimensional scaling (nMDS) plot of *D. pumilio* alkaloid profiles for individual frogs in seven populations on Isla Bastimentos during the wet season. nMDS plot is based on major, minor, and trace alkaloids. Each symbol represents an individual frog from a specific population. Site numbers correspond to map in Fig. 1. The size of each circle is proportional to the total number of alkaloids in that individual

zidines, and 2,5-disubstituted decahydroquinolines have been identified in ants of the subfamily Myrmicinae (Daly et al., 1994a, 2000; Jones et al., 1999; Clark et al., 2005). These alkaloids contain unbranched carbon skeletons. In addition, 3,5-disubstituted lehmizidines, histrionicotoxins, and gephyrotoxins are alkaloid classes that share certain structural features (i.e., unbranched carbon skeletons) with those of known myrmicine ant alkaloids, and it is expected that they will prove to be of myrmicine ant origin as well (Daly et al., 2005). 5,8-Disubstituted indolizidines have been identified in a mixed collection of leaf-litter arthropods from Isla Bastimentos, Panama (Daly et al., 2002), and recently in ants of the subfamily Myrmicinae from Madagascar (Clark et al., 2005). 2,5-Disubstituted pyrrolidines and 3,5-disubstituted pyrrolizidines were recently reported in ants of the subfamilies Formicinae and Ponerinae from Madagascar (Clark et al., 2005). A 5,6,8-trisubstituted indolizidine has been identified in ants from Panama; however, the sample included several ant species, and the specific ant taxon that contains the compound could not be determined (Saporito et al., 2003). In addition, a 5,6,8-trisubstituted indolizidine and a 1,4-disubstitued quinolizidine have been identified in an oribatid mite (Takada et al., 2005). Pumiliotoxin alkaloids have been identified in ants of the subfamily Formicinae from Panama (Saporito et al., 2004) and more recently in oribatid mites (Takada et al., 2005).

Poison frogs' dependence on arthropods for chemical defense suggests that much of the geographic and temporal variation observed in alkaloid profiles is the result of variation in availability of alkaloid-containing arthropods. In tropical regions, arthropod abundances are known to vary spatially and temporally (Janzen and Schoener, 1968; Janzen, 1973; Lieberman and Dock, 1982; Levings and Windsor,

1984; Levings, 1983), suggesting that the availability of alkaloid-containing arthropods is variable. On Isla Bastimentos, the spiropyrrolizidine alkaloid **236** and the pumiliotoxin alkaloids **307A** and **323A** are major alkaloids in certain populations of *D. pumilio*. Recently, arthropod sources for these two classes of alkaloids have been shown to occur sympatrically with the same populations of *D. pumilio* examined in this study (Saporito et al., 2003, 2004). The spiropyrrolizidine **236** is a major alkaloid in two of the seven populations of *D. pumilio* on Isla Bastimentos; however, in 1972 it was not detected in these populations (Daly et al., 1987, 2003). The appearance of this alkaloid in populations of *D. pumilio* today is most likely due to a recent introduction and current availability of the millipede *Rhinotus purpureus* (Family: Siphonotidae) as a dietary source (Saporito et al., 2003). The presence of **236** in certain populations of *D. pumilio* supports the idea that spatial and temporal differences in arthropod availability plays an important role in alkaloid variation of these dendrobatid frogs. Recently, a variety of different spiropyrrolizidine alkaloids, including **236**, have also been discovered in *R. purpureus* millipedes from Madagascar, which occur sympatrically with alkaloid-containing mantellid frogs (Clark et al., 2005). These findings suggest that variation in alkaloids within the same species of arthropod may also be responsible for alkaloid variation among poison frogs. The pumiliotoxins **307A** and **323A** are present in variable amounts in certain populations of *D. pumilio* on Isla Bastimentos. Dietary sources for these have been identified in two different genera of ants in the Subfamily Formicinae (*Paratrechina* and *Brachymyrmex*) in regions where *D. pumilio* populations are known to contain variable amounts of these alkaloids (Saporito et al., 2004). Interestingly, their presence in *Brachymyrmex* is also variable. The pumiliotoxins **307A** and **323A** were not detected in all *Brachymyrmex* samples collected within a site, and certain ant samples contained only one of the two pumiliotoxins (Saporito et al., 2004). The pumiliotoxins were not detected in ants from the dry season (Saporito et al., 2004). The variable presence of pumiliotoxins in *Brachymyrmex* suggests the possibility of a dietary source or microsymbiont as the ultimate source for these alkaloids in ants (Saporito et al., 2004). Recently, the pumiliotoxins **237A** and **251D** and the deoxypumiliotoxin **193H** were identified in two different species of scheloribatid mites (*Scheloribates azumaensis* and *Scheloribates* sp.), suggesting that mites may be the ultimate source for some of the pumiliotoxin alkaloids (Takada et al., 2005). However, ants in the genus *Solenopsis* are known to be caste-specific in the production of alkaloids (Deslippe and Guo, 2000; Torres et al., 2001), and it remains possible that alkaloid variation in *Brachymyrmex* is attributable to differences among castes. Regardless of the source of alkaloid variation in *Brachymyrmex*, the variable presence of pumiliotoxins in these ants suggests that alkaloid variability of certain arthropod prey items may contribute to alkaloid profile variation in *D. pumilio*.

Other factors may also contribute to alkaloid variation within and among populations of *D. pumilio*. Some poison frogs possess the ability to modify certain dietary pumiliotoxin alkaloids (Smith et al., 2002; Daly et al., 2003). One species in the genus *Pseudophryne* is able to convert the pumiliotoxin **307A** to a significant extent into reduced and/or hydroxylated pumiliotoxins of molecular weights 309, 323, and 325 (Smith et al., 2002). Frogs in the genus *Dendrobates* are able to convert over 70% of the natural dietary (+)-enantiomer of pumiliotoxin **251D** (by stereoselective hydroxylation) into the natural (+)-allopumiliotoxin **267A** (Daly et al., 2003). Frogs of the genus *Dendrobates* did not hydroxylate the unnatural (−)-en-

antiomer of pumiliotoxin **251D** (Daly et al., 2003). The presence of such a hydroxylase raises the likelihood that poison frogs are able to convert other pumiliotoxin alkaloids (i.e., side-chain hydroxylated analogs) into allopumiliotoxins. Both of these processes are likely mediated by enzymes and are under some genetic control, and a differential ability to modify certain alkaloids would influence variability in profiles. Pumiliotoxin **251D** is always accompanied by **267A** in populations of *D. pumilio* that have been examined (this study; Daly et al., 1987, 2003, and unpublished data), and, therefore, the data do not suggest a difference in the ability to convert **251D** within and among populations of *D. pumilio*. However, differences among genera of poison frogs in the ability to modify certain pumiliotoxins appear to contribute to alkaloid variation among species (Daly et al., 2003). For instance, frogs of the genera *Phyllobates* and *Epipedobates* did not hydroxylate dietary pumiliotoxin **251D** (Daly et al., 2003). The ability to uptake and sequester certain alkaloids is also likely under genetic control (Myers et al., 1995), and variation in alkaloid profiles of *D. pumilio* may be in part due to genetic variation in the uptake/sequestration process. Although not intended to address genetic differences in alkaloid uptake, Summers et al. (1997) demonstrated that there were low degrees of mitochondrial DNA divergence among populations of *D. pumilio* in the Bocas del Toro region of Panama, including populations on Isla Bastimentos. This study illustrates that there are no prominent genetic differences among populations, at least as revealed by mitochondrial markers, suggesting that genetic factors are not responsible for the variation observed in alkaloid profiles. In addition, genetic factors would not solely explain differences in alkaloid profiles between seasons. Finally, it remains possible that differences in prey selectivity may explain alkaloid variation. If different populations and individuals within populations are differentially utilizing prey, alkaloid profiles could reflect variable prey consumption. In a study of *D. pumilio* diet in Costa Rica, Donnelly (1991) suggested that frogs change their diet over time in response to natural fluctuation in the availability of food. It is likely that differences in diet of *D. pumilio* within and among populations are also attributable to differences in the availability of arthropod prey. Although differences in selectivity may account for some of the alkaloid variation observed among different species of poison frogs, it is unlikely that individuals of *D. pumilio* confined to a small area exhibit such marked geographic and seasonal differences in dietary selectivity. Donnelly (1991) also reported differences in diet between juveniles and adults as well as between sexes of *D. pumilio*. Here, we only examined adults. However, both males and females were examined, and it is possible that some of the individual variation within populations is related to sexual differences in diet. Although males and females were collected from each population in this study, sample sizes were not large enough to statistically examine differences between sexes.

The natural diet of *D. pumilio* consists mainly of ants and mites (Donnelly, 1991; Caldwell, 1996; Saporito et al., unpublished data). In general, dendrobatid frogs of the genera *Dendrobates*, *Epipedobates*, and *Phyllobates* consume a large proportion of ants as part of their diet, and members of the genus *Dendrobates* (including *D. pumilio*) and some members of the genus *Epipedobates* have been referred to as "ant-specialists" (Donnelly, 1991; Toft, 1995; Caldwell, 1996). Frogs of the genera *Mantella*, *Melanophryniscus*, and *Pseudophryne* have also been shown to consume large amounts of ants (Pengilley, 1971; Filipello and Crespo, 1994; Vences et al., 1998). These findings have led many researchers to suggest that myrmecophagy has

played a major role in the evolution of alkaloid sequestration in poison frogs (Toft, 1995; Caldwell, 1996; Vences et al., 1998, 2003; Summers and Clough, 2001; Daly et al., 2002; Santos et al., 2003; Saporito et al., 2004; Clark et al., 2005; Darst et al., 2005). Interestingly, although myobatrachid frogs of the genus *Pseudophryne* consume a large proportion of ants, they do not appear to accumulate myrmicine ant alkaloids (see Smith et al., 2002). Instead, theses frogs synthesize unique indolic pseudophrynamine alkaloids (Smith et al., 2002). Therefore, their uptake system may be designed to retain the pseudophrynamines and also to accept certain dietary pumiliotoxins for uptake and storage (see Smith et al., 2002). In our study, many of the alkaloids are from classes known or suspected to be of ant origin, suggesting that myrmecophagy plays a dominant role in chemical defense in these frogs. However, certain tricyclic, indolizidine, quinolizidine, spiropyrrolizidine, and pumiliotoxin alkaloids were recently detected in mites (Takada et al., 2005; Saporito et al., unpublished data), suggesting that mites represent another major source of alkaloids in poison frogs.

In summary, individual alkaloid profiles of *D. pumilio* are shown to vary among populations and between the dry and wet season on Isla Bastimentos, Bocas del Toro, Panama. Variation in profiles among populations is related to geographic proximity, and it is likely that the availability of alkaloid-containing arthropods in this region share a similar distribution. Individual frogs within a population have alkaloid profiles more similar to each other than to frogs from different populations. Individual variation within a population is attributed to small geographic differences in the distribution of alkaloid-containing prey. Overall, alkaloid variation in *D. pumilio* is likely the result of spatial and temporal variation in the availability of certain alkaloid-containing arthropods and the presence of alkaloids in these arthropods. Many of the alkaloids found in *D. pumilio* appear to be from ant sources, further indicating the importance of ants in chemical defense among poison frogs. Recent findings that certain frog skin alkaloids are also known in mites, suggests that mites may also play an important role in the chemical defense of poison frogs.

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