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Individual Variation in Alkaloid Content of Poison Frogs of Madagascar (*Mantella*; Mantellidae)

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Abstract Brightly colored Malagasy poison frogs, *Mantella* spp., sequester lipophilic, basic alkaloids from arthropod prey for their own chemical defense. Consequently, microsympatric prey diversity is expected to influence alkaloid diversity observed in poison frogs. Twenty-two specimens of three *Mantella* species from four localities in moist forests of southeastern Madagascar were analyzed individually via gas chromatography-mass spectrometry, revealing that they contain over 80 known alkaloids. Frogs within a locality possessed significantly similar alkaloid content and diversity, while frogs from areas that varied in disturbance, elevation, and/or species showed greater differences. Based on dietary data, the larger frog species *Mantella baroni* consumed more and larger prey, and showed greater diversity in skin alkaloids than significantly smaller *Mantella bernhardi*. Additionally, frogs from the most pristine locality had the greatest number of alkaloids, whereas individuals from the most disturbed localities had the least. In a comparison of frog alkaloid profiles over a 10- to 14-yr period, alkaloid turnover, and thus presumably alkaloid-source arthropod turnover, was high in a disturbed locality and low in the pristine primary forest locality. We demonstrate that the nonlethal transcutaneous amphibian stimulator (TAS) is effective for harvesting alkaloids from poison frogs; future studies using this device could obtain larger sample sizes without harming local frog populations.

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Keywords Chemical defense · Allomones · *Mantella* · Chemical ecology · Temporal variation · Geographic variation · Habitat disturbance · Ant alkaloids · Alkaloid profiles · Poison frogs

Abbreviations

TAS	transcutaneous amphibian stimulator
Ampa	Ampasimpotsy
Vato	Vatoharanana
Saha	Sahavondrona
Vohi	Vohiparara
AMNH	American Museum of Natural History
UADAB	University of Antananarivo Department of Animal Biology
RA	relative abundance in EI mode
SVL	snout–vent length

Introduction

Frogs that are able to sequester alkaloids into skin from arthropod prey possess an important evolutionary advantage; they not only afford themselves chemical protection without expending energy to produce such defensive compounds, but also exploit a nutrition niche that other species may reject as bitterly distasteful and dangerous (dietary data not shown). Such ingested alkaloids are known nematicides, insecticides (including antimosquito), and neurotoxins, and provide these frogs with protection against a wide range of possible foes (Daly et al., 1999, 2005 and references therein). More than 800 lipophilic alkaloids from 24 classes, designated in boldface by molecular weight and distinguishing letter (e.g., **251O**; Fig. 1) (Daly et al., 2005), have been documented in certain frog genera from five families. Most genera within these families do not have alkaloids, and thus the traits for alkaloid sequestration and storage in skin glands, along with specialization on ants, has likely arisen multiple times in evolution (Clark et al., 2005 and references therein).

Such poison frogs include *Mantella* of Madagascar (Mantellidae), *Dendrobates*, *Epipedobates*, and *Phyllobates* of Central and South America (Dendrobatidae), *Melanophryniscus* of South America (Bufonidae), *Pseudophyrne* and, to some extent, *Geocrinia* of Australia (Myobatrachidae; see Smith et al., 2002), and to a lesser extent, one *Limnonectes* species of Thailand (Ranidae; see Daly et al., 2004, 2005). Multiple feeding experiments with certain captive-born poison frogs revealed that: (1) dietary alkaloids are usually sequestered unchanged; (2) some chemical modifications of ingested alkaloids exist; and (3) sequestered alkaloids are retained in frogs' skin glands for several years (Smith et al., 2002; Daly et al., 1997, 2003). Many of these "poison frog alkaloids" are also known from tropical and/or temperate ants, mites (Takada et al., 2005), millipedes, beetles, and plants (reviewed in Eisner et al., 1978; King and Meinwald,1996; Leclercq et al., 2000; Daly et al., 2005; Laurent et al., 2005). In Madagascar, a siphonotid millipede and three ant subfamilies, some of which were confirmed frog prey, contained alkaloids in common with sympatric *Mantella* (Clark et al., 2005); however, putative sources of some of these same alkaloids occur in different ant subfamilies in the Neotropics (see Jones et al., 1999). Further related research on occurrence and pharmacology of alkaloids in poison frogs and their prey is reviewed in Daly et al. (1999, 2005), Saporito et al. (2004), Clark et al. (2005) and Daly (2005).

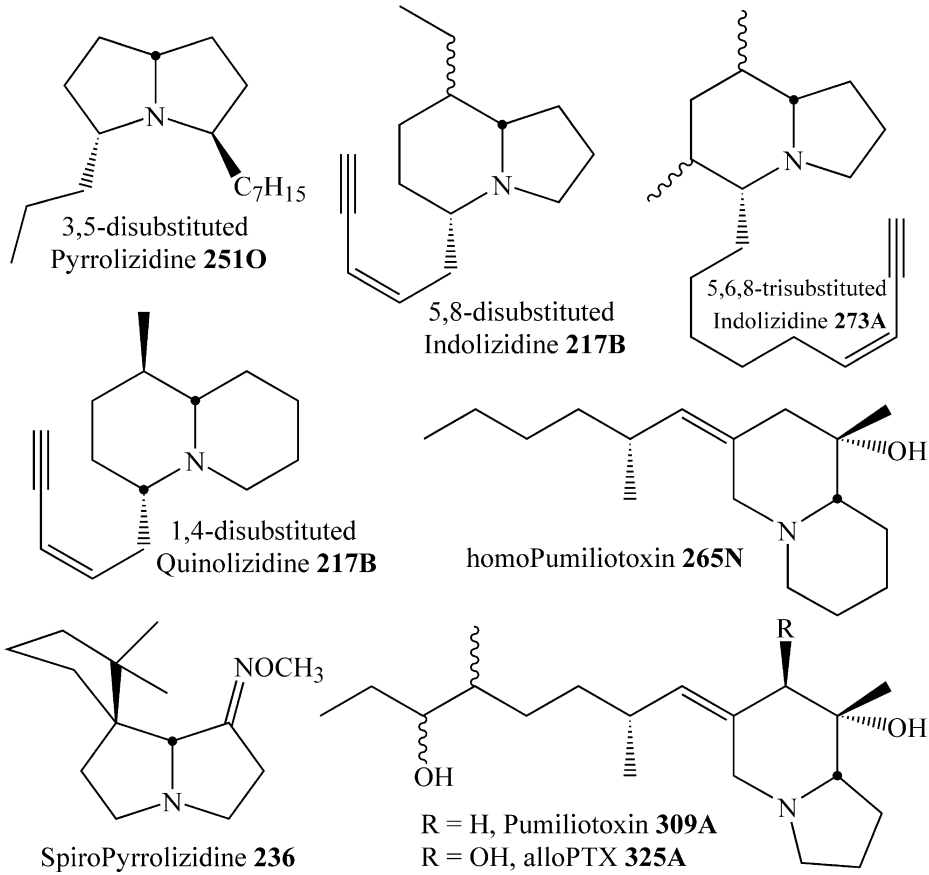

Fig. 1 Representative structures of select minor and major alkaloids detected in *Mantella* frogs of the Ranomafana region, suspected or confirmed to be of arthropod origin

Daly (1982; see Table 22) and his colleagues (Myers and Daly, 1980; Daly et al., 1990, 1992, 1994a, b, 1996, 2002) have demonstrated that the alkaloid content of pooled frog skins vary by population/locality. Myers et al. (1995) reported variability between frogs, with two individual *Dendrobates granuliferus* skins at each of three locales in Costa Rica; also, at one of these locales two individual and one 30-skin samples of microsympatric *D. pumilio* tentatively revealed that the two species are similar in alkaloid content. Saporito et al. (2006) also discuss variation in alkaloids recovered from 70 individual *D. pumilio* skins. Mebs et al. (2005) made methanolic extracts from 81 individual *Melanophyrniscus montevidensis* toads from Uruguay, to reveal by quantitative gas chromatography-mass spectrometry (GC-MS) that there was variation among individuals and populations in the amount of both pumiliotoxin **251D** and the nonalkaloid phenolic hydroquinone.

We previously reported that within a locality, sympatric individuals of two *Mantella* species had similar alkaloid profiles (see supporting information online and Table 1 in Clark et al. 2005). Here, we explore patterns of variation for >80 alkaloids detected in individual *Mantella* poison frogs. Individual variation within or among poison frog species

of a region may reveal important aspects of community ecology; thus, we report the detailed alkaloid content (profiles) of 22 individual *Mantella* frogs of three species sampled from four localities in the Ranomafana region of southeastern Madagascar (Fig. 2), alongside data on frog size and "snapshot" prey consumption. Alkaloid profiles from *M. baroni* collected at two of these localities in 1989 and 1993 are compared to frogs from the 2003 expedition.

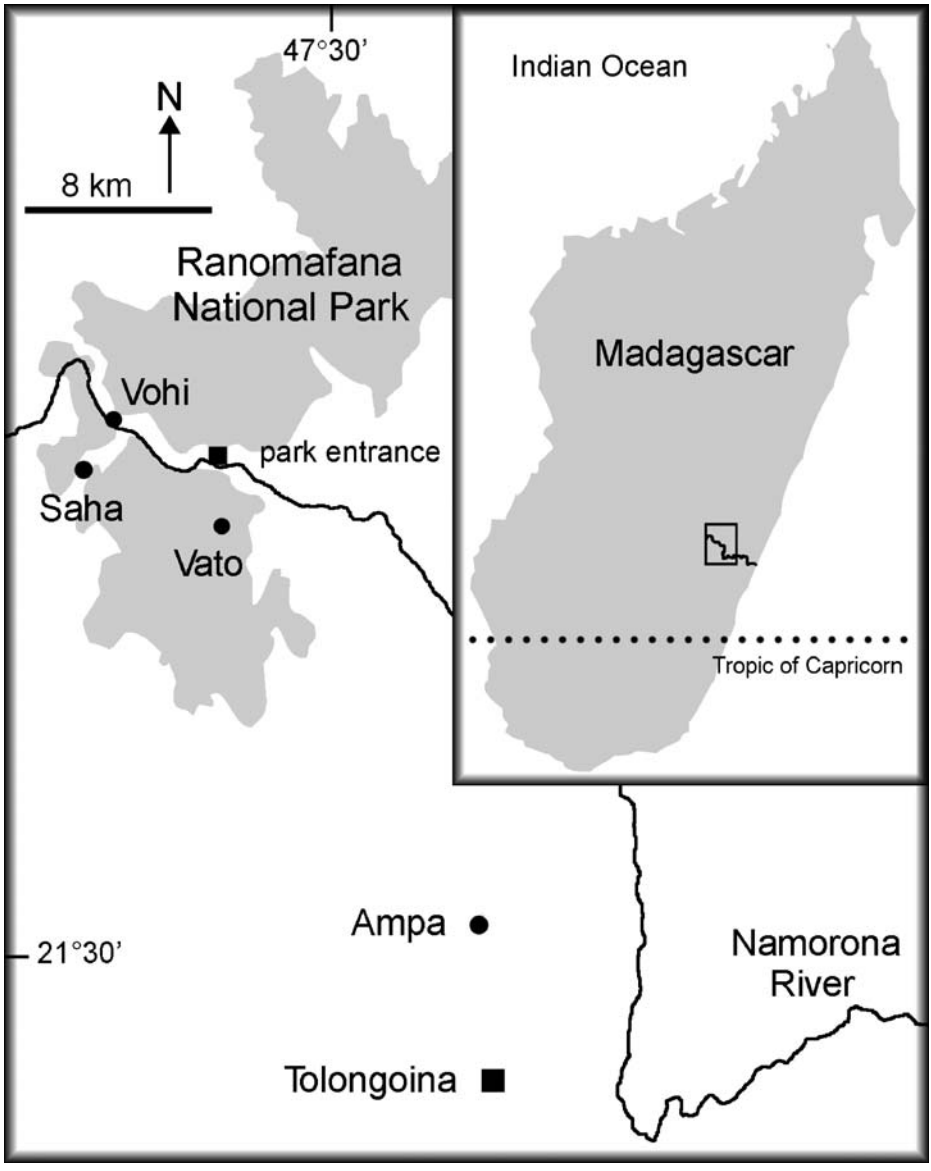

Fig. 2 *Mantella* individuals were sampled in and near Ranomafana National Park at riparian locales indicated by solid circles (after map in Bradt, 1999)

Methods and Materials

Field Collections

Materials and methods are described in detail in Clark et al. (2005). Briefly, riparian *Mantella madagascariensis*, *M. baroni*, and *M. bernhardi* were collected in March and April 2003 at localities in moist forests of the Ranomafana region in southeastern Madagascar (Fig. 2). Localities included Ampasimpotsy (Ampa, disturbed forest fragments, 550 m; 21° 28.796′S, 47°33.424′E), Vatoharanana (Vato, old-growth primary forest, 1100 m; 21°17.444′ S, 47°25.569′E), Sahavondrona (Saha, disturbed roadside forest, 1100 m; 21°15.450′S, 47° 21.609′E), and both sides of the Kidonavo stream at Vohiparara (Vohi 1 and 2, disturbed roadside forest, 1100 m; 21°13.587′S, 47°22.193′E). Ampa is 25–34 km away from the other three localities, which are clustered 4–9 km from one another within Ranomafana National Park (Fig. 2). In addition, temporal variations in the alkaloid content of *M. baroni* were assessed with samples collected at Vato (December 1989, 10 combined skins) and Saha (January 1993, 17 combined skins); these two samples were provided by John W. Daly (National Institutes of Health), and previously described in Daly et al. (1996).

Sample Preparation and Analyses by Gas Chromatography-Mass Spectrometry

Eighteen *Mantella* frogs were skinned into methanol, and alkaloid fractions were made following the methods of Daly et al. (1994b). These and other frog voucher specimens were deposited and the stomach contents removed at the American Museum of Natural History (AMNH, A168355–A168393) and the University of Antananarivo, Department of Animal Biology (UADAB #1–8). Additional exudates were collected from four live frogs by using a TAS (purchased from Jacqualine Grant, and described in Grant and Land, 2002) and a methanol-laced Kim-wipe; these frogs were rinsed in water and released unharmed. Skin alkaloid fractions and TAS extracts were individually analyzed for alkaloids on a Micromass™ GC-time of flight (TOF)-MS in CI (with 100% NH_3) and EI modes with a ramp of 10°C/min from 100 to 280°C, with oven maintained at 100°C for the first 5 min and at 280°C for the final 5 min. The MS libraries of Daly et al. (1999, 2005) were used to identify over 80 known alkaloids in *Mantella* sampled at Ranomafana in 2003, and the two *M. baroni* standards of Daly et al. (1996) provided retention times of known alkaloids for comparison. Based only upon their relative abundance (RA) in EI mode, these alkaloids are reported in Table 1 as trace (RA < 10%), minor (10% < RA < 70%), or major alkaloids (RA > 70%; see chromatograms in Supplementary Information); although not absolutely quantitative, any possible ionization biases should be consistent among all samples tested.

Statistical Analyses

Statistics were performed with SPSS v. 13. A *t*-test was used to compare the mean alkaloid diversity of Vohi *M. baroni* sampled by TAS vs. full-skin extraction, and to compare means of both frog size [snout–vent length (SVL)] and number of prey between *M. bernhardi* and *M. baroni*. A linear regression was used to evaluate the relationship between frog size and number of prey within the Vohi locality. A *partial correlation* for all *Mantella*,

Table 1 Alkaloids detected in *Mantella* frog individuals[a] of the Ranomafana region, southeastern Madagascar

Montella spp.		*M. baroni*								*M. md.*	*M. baroni*					*M. bernhardi*										Family[b]
Locality		Vohiparara 1				Vohiparara 2					Sahavondrona					Vatoharanana				Ampasimpotsy						World[b]
Alkaloid class	MW code	U1	U2	82	L2	L3	L4	U3	83	U4	D[a]	84	57	U5	U6	D[a]	87	88	89	90	91	92	93	U8	U7	Daly et al.[b]
Polyzonamine	**151B**	−	−	−	−	−	−	−	+	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	M, D
Unclass	**155**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D
Nicotine	**162**	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D
Precoccinelline	**193C**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	M, D, B
3,5-I	**195B**	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D, B
Pyr	**197B**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	D
Pip	**197E**	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	D
5,8-I	**203A**	−	−	−	−	−	−	−	+	⊕	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	M, D
5,8-I	**205A**	−	−	−	−	−	−	−	+	⊕	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	M, D
5,8-I	**207A**	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	M, D, B
Tricyclic	**207J**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	M, D
Unclass	**207N**	+	+	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D
5,8-I	**209B**	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D
5,8-I	**209I**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	M, D
1,4-Q	**217A**	−	⊕	⊕	+	+	+	⊕	⊕	⊕	+	+	−	+	+	⊗	⊗	⊗	⊗	+	⊕	−	⊕	⊕	⊕	M
5,8-I	**217B**	+	⊕	⊕	+	⊕	+	+	⊕	⊕	⊕	⊕	⊕	⊕	⊗	⊗	⊗	⊗	⊗	+	−	−	+	+	⊕	M, D
5,8-I	**217B′**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	⊗	⊕	M, D?
5,8-I	**217B″**	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D?
5,8-I	**219F**	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	⊕	⊕	⊕	⊕	M, D
5,8-I	**219L**	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M
5,8-I	**221I**	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	⊕	⊕	⊕	⊕	M
5,8-I	**221I′**	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	×
SpiroP	**222**	−	−	−	−	−	−	−	−	⊕	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D
3,5-P	**223B**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	⊕	⊕	⊕	−	M, D, B
Izidine	**223C**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	M, D
3,5-P	**223H**	+	+	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D, B

3,5-P	**223H′**	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	M, D, B?
3,5-P	**223H″**	⊕	−	−	−	−	−	+	⊕	⊕	−	−	−	−	−	−	−	−	−	+	⊕	−	+	+	+	×
3,5-P	**223H‴**	+	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	×
5,8-I	**223I**	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D, B
1,4-Q	**231A**	+	⊕	⊕	+	⊕	+	+	⊕	−	⊕	⊗	⊗	⊕	⊕	⊕	⊕	⊕	⊕	−	−	−	−	−	−	M, D
1,4-Q	**231A′**	−	+	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	M, D?
Unclass	**231J**	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D
1,4-Q	**233A**	−	−	⊕	+	⊕	−	−	−	−	⊕	−	+	−	−	⊕	⊕	⊕	⊗	⊗	⊕	⊗	⊕	⊕	⊗	M, D
Unclass	**235R**	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D
SpiroP	**236**	−	−	−	−	−	−	−	+	⊕	+	−	−	+	+	−	−	−	−	−	−	−	−	−	−	M, D, B
SpiroP	**236′**	−	−	−	−	−	−	−	+	⊕	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	×
PTX	**237A**	+	+	+	+	−	+	−	−	−	+	−	−	−	−	⊗	⊗	⊗	⊗	−	−	−	+	−	−	M, D
3,5-P	**237G**	−	−	−	−	−	−	−	−	⊕	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D, B
3,5-P	**237G′**	−	−	−	−	−	−	−	−	⊕	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D, B?
SpiroP	**238**	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	D
3,5-P	**239K**	−	−	−	−	−	−	−	−	⊕	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M
3,5-P	**239K′**	−	−	−	−	−	−	−	−	⊕	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M
5,8-I	**241F**	−	⊕	−	−	−	−	−	−	−	+	⊕	+	⊕	−	−	−	−	−	−	−	−	−	−	−	M, D
5,8-I	**243B**	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	M, D
5,8-I	**243C**	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	M
5,8-I	**243D**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	⊕	⊕	⊕	⊕	−	−	−	−	−	−	M
5,8-I	**245B**	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	⊕	+	⊕	M
5,8-I	**245C**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	⊕	⊕	⊕	⊕	⊕	−	−	⊕	+	⊕	M
DHQ	**245E**	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D
5,8-I	**247E**	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	⊕	M, D
3,5-I	**249A**	+	−	+	+	+	−	−	−	−	−	+	−	−	−	−	+	+	+	−	−	−	−	−	−	M, D
3,5-I	**249A′**	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	M, D
PTX	**251D**	⊗	⊗	⊗	⊗	⊕	⊕	⊕	⊕	⊕	⊕	−	−	−	−	+	⊕	⊕	⊗	−	−	−	−	−	−	M, D, B
DeoxyPTX	**251H**	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D
5,6,8-I	**251M**	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D
5,8-I	**251N**	−	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D
3,5-P	**251O**	⊕	⊗	⊗	⊕	+	+	+	⊕	+	⊕	⊕	⊕	⊗	⊗	+	⊕	⊕	⊗	⊗	⊗	⊗	⊗	⊕	⊕	M
hPTX	**251R**	+	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D

Table 1 (continued)

Montella spp.		*M. baroni*								*M. md.*	*M. baroni*									*M. bernhardi*						Family[b]
Locality		Vohiparara 1				Vohiparara 2					Sahavondrona					Vatoharanana				Ampasimpotsy						World[b]
Alkaloid class	MW code	U1	U2	82	L2	L3	L4	U3	83	U4	D[a]	84	57	U5	U6	D[a]	87	88	89	90	91	92	93	U8	U7	Daly et al.[b]
SpiroP	**252A**	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D
Izidine	**255B**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	⊕	−	−	−	−	−	−	M, D
1,4-Q	**257D**	−	−	−	−	−	+	−	+	−	−	+	−	+	−	+	+	⊕	⊕	−	−	−	−	−	−	M, D
Tricyclic	**261C**	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M
Unclass	**265F**	−	−	−	−	−	−	−	−	−	⊕	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M
hPTX	**265N**	⊗	⊗	⊗	⊕	⊕	⊕	⊗	⊗	⊗	⊗	⊕	+	−	⊕	−	−	−	−	−	−	−	−	−	−	M, D, B
PTX	**267C**	−	−	−	−	−	−	−	−	⊗	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M,D,B,Myo
Unclass	**271B**	−	−	+	−	+	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	M,D
3,5-I	**271F**	−	−	−	−	−	−	−	−	−	⊕	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M
5,6,8-I	**273A**	⊗	⊗	⊗	⊕	+	⊕	⊕	⊕	−	⊕	⊕	+	−	+	−	−	−	−	−	−	−	−	−	−	M
5,6,8-I	**273A′**	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	M
3,5-I	**275C**	+	+	+	⊕	⊕	+	+	−	−	+	+	−	−	−	+	⊕	+	⊕	−	−	−	−	−	−	M, D
3,5-I	**275C′**	+	⊕	⊕	⊕	−	⊕	⊗	+	+	−	⊕	−	+	−	−	⊕	⊕	⊗	−	−	−	−	−	−	M, D
5,6,8-I	**275E**	−	−	−	−	−	−	−	⊕	⊕	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D
DeoxyPTX	**281G**	−	−	−	−	−	−	−	−	−	⊕	−	−	−	−	−	−	−	−	−	−	−	−	−	−	D
DeoxyPTX	**291E**	+	−	+	−	−	−	−	−	−	+	−	−	−	−	⊕	+	+	−	−	−	−	−	−	−	M
PTX	**291G**	−	−	−	−	−	−	−	⊕	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D, B
DeoxyPTX	**293B**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	M
5,6,8-I	**293C**	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M, D
DeoxyPTX	**293D**	⊕	⊕	+	−	−	−	−	−	−	⊕	−	−	−	−	+	+	+	⊕	−	−	−	−	−	−	M
PTX	**305B**	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M
PTX A	**307A**	−	−	−	−	−	−	−	−	−	⊗	−	−	−	−	+	−	−	+	−	−	−	−	−	−	M, D
PTX	**307D**	−	−	−	−	−	−	−	−	⊕	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	D
PTX	**307F″**	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	+	⊕	−	−	−	−	−	−	M, D
PTX	**307F‴**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	⊕	−	−	−	−	−	−	M, D
PTX	**307G**	⊕	⊗	⊗	−	−	+	⊕	⊗	+	+	⊕	−	⊕	⊕	⊕	⊗	⊕	⊕	−	−	−	−	−	−	M, D

PTX	**309A**	⊗	⊗	⊗	⊕	⊗	⊕	⊗	⊗	+	⊕	+	⊕	−	⊗	⊕	⊕	+	+	−	+	−	−	−	−	M, D
PTX	**309C**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	⊕	−	−	−	−	−	−	−	−	−	D
aPTX	**323B**	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	+	⊕	⊕	⊕	+	⊕	+	⊕	M,D,B,Myo
hPTX	**323E**	−	+	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	M
aPTX	**325A**	−	+	+	−	+	+	⊕	−	−	⊕	+	+	−	+	+	⊕	⊕	⊕	⊕	+	+	+	+	⊗	M, D, Myo
Total # of alkaloids		20	23	21	15	13	19	17	26	28	30	25	12	10	11	27	26	29	31	11	11	8	14	14	14	
Avg. # alkaloids/frog/locality		19.8 ± 3.4			20.2 ± 4.9			20.6 ± 6.3				14.5 ± 7.0				28.7 ± 2.5						12.0 ± 2.4				
Total # of alkaloids/locality		31(Vohi 1)			59(both)			49(Vohi 2)			30				28	27			33						20	
K-means Cluster Group		A	A	A	A	A	A	A	B	B	A	C	C	C	C	D	D	D	D	C	C	C	C	C	C	
TAS (T) or Skin (S)		S	S	S	T	T	T	S	S	S	S	S	T	S	S	S	S	S	S	S	S	S	S	S	S	
No. prey recovered[c]		47	48	33	nd	nd	nd	21	66	13	nd	27	23	41	30	nd	30	40	10	16	27	16	7	36	4	
Snout–vent length[c] (mm)		27	24	24	24	27	25	22	30	22	nd	27	25	22	28	nd	26	24	27	17	22	20	21	22	19	

M. md: *Mantella madagascariensis* (single specimen = U4); D: Daly's samples; −: not detected; +: trace alkaloid [(< 10% relative abundance (RA) in EI mode]; ⊕: minor alkaloid (10% < RA < 70%); ⊗: major alkaloid (RA > 70%); nd: no data; prime symbol (′) indicates isomer of a different GC retention time. Alkaloid classes (see Fig. 1): #,# substituted...P: pyrrolizidine; I: indolizidine; Q: quinolizidine; Pyr: pyrrolidine; Pip: piperidine. (a, h)PTX: (allo, homo)pumiliotoxin; SpiroP: spiropyrrolizidine; DHQ: decahydroquinoline; Unclass: no class assigned. Individual *Mantella* frogs are coded with voucher identification numbers, as U# for UADAB deposited *Mantella* #s 1-8, and as ## for AMNH deposited frogs A1683## (A168357-A168393). L# refers to live frogs whose exudates had been collected with a transcutaneous amphibian stimulator, photographed, and then released unharmed.

[a] Our analyses of *M. baroni* samples provided by Daly for Saha (January 1993, 17 skins) and Vato (December 1989, 10 skins); see Daly et al. (1996).

[b] Data from Daly et al. (1996, 1999, 2005)—the latter includes data from Clark et al. (2005). Occurrence of isomers was pooled. Frog families: M, Mantellidae (Madagascar); D, Dendrobatidae (Central and South America); B, Bufonidae (South America); Myo, Myobatrachidae (Australia); ×, not included in Daly et al. (2005).

[c] A *M. bernhardi* specimen (A168358) from Ampa not analyzed for alkaloids measured 18 mm, and 10 arthropods were recovered from its stomach.

controlled for by locality, was used to evaluate the relationship of SVL with both the number of prey and individual frog skin-alkaloid diversity. One-way analysis of variance (ANOVA) was used to compare mean alkaloid diversity per frog among all four localities, among the three localities of constant (1100 m) elevation, and between frogs of the two elevation groups. *K*-Means cluster analysis was performed on 22 *Mantella* frogs by using all alkaloids requesting four clusters, and was followed by a cross-tabulation between the cluster membership and the localities.

Results and Discussion

Variation in Alkaloid Content of Individual Frogs, within and among Localities

Cluster analysis revealed that the three *M. baroni* and 1989 standard from Vato represent a distinct group based on the specific alkaloid content of the resident individual frogs (see Cluster groups at bottom of Table 1), indicating that frogs within a locality are more similar to one another compared to frogs from different localities. Seven of nine frogs from both sides of the Kidonavo stream at Vohi clustered in another group of individuals, indicating little variation at this fine spatial scale (Table 1). The other two Vohi frogs clustered in a third unique group, demonstrating little difference in alkaloid profiles of microsympatric *Mantella* species. Frog individuals from the two most disturbed locales clustered exclusively in another group, including all four frogs from moderately disturbed Saha and all six frogs from the disturbed forest fragments of Ampa; these two localities also had the lowest mean number of alkaloids per frog and the six individuals with the fewest alkaloids (Table 1).

Out of the 91 alkaloids in Table 1, 26 were detected in only a single frog individual, 16 were detected in multiple frogs from a single locality, and 11 were detected in multiple individuals from two localities. Vohi 1 and 2 are separated only by the 2- to 4-m-wide Kidonavo stream, and frogs from these sites contained many alkaloids in common (e.g., **207N**, **251D**, **265N**, **273A**); 21 alkaloids were present on both sides of the Kidonavo stream, and the other 38 were only detected in frogs from either Vohi 1 or Vohi 2 (Table 1; of these 38 alkaloids, 25 were found only in a single frog). Of the 91 alkaloids, 26 were detected in only one of the various 22 frogs sampled, and may represent rare prey. The fact that Vohi samples contain 25 of these 26 unique (in one of 22 frogs) alkaloids suggests this intermediately disturbed site may have an elevated number of rare arthropod-source species. Alkaloids appearing in more than one frog that are limited to one locality include: **217B** and **223B** at Ampa; **151B**, **195B**, **207N**, **251R**, and **275E** at Vohi; **273A′** at Saha; and **207J**, **209I**, **223C**, **223M**, **243D**, **255B**, **307F″**, and **307F‴** at Vato. These results also suggest that frogs within a locality are more similar in terms of alkaloid content compared to frogs from different localities. Alkaloids that appear in the majority of frogs from only two localities in 2003 include **203A**, **205A**, **207A**, **219F**, **221I**, **223H″**, **241F**, **245C**, **265N**, **291E**, and **293D** (Table 1).

The distribution and amount of particular alkaloids in individual *Mantella* frogs should reflect how abundant the leaf-litter source arthropod is in the environment. Although frog sequestration efficiency varies among alkaloid classes (see Daly et al., 2003), such sequestration biases should be constant among *Mantella* sampled in this study. For example, alkaloids such as **251O** and **217B** were found in all or most of the 22 individuals (Table 1, Fig. 1); these alkaloids are likely common in frogs because the source arthropod is particularly abundant in the region, so it was not surprising that the endemic ant source species for these common alkaloids was discovered (Clark et al., 2005). As-yet-undetermined arthropod sources for **217A**, **233A**, and **325A** are also likely widely distrib-

uted across the entire region, whereas sources of **231A**, **275C**, and **307G** appear limited to localities within Ranomafana Park boundaries (see Table 1). Ampa is relatively far from the three 1100-m localities and has an elevation of only 550 m. This suggests that the arthropod sources of **231A**, **275C**, and **307G** could be limited to higher elevations. In fact, previous work indicates ant species assemblages and diversity do vary with elevation (Fisher, 1998).

Ants were the most numerous taxon in *Mantella* stomach samples (>67%), and individuals of the same ant species were often collected in the same stomach sample (Table 4 of Clark et al., 2005; unpublished data of V.C.C. and B.L.F.). This is not surprising, because ants are colonial and locally abundant. Based on this data, and the fact that most of the more commonly distributed and "major/minor" alkaloid classes reported here are known from some ant source, the majority of common "minor/major" alkaloids in Table 1 are suspected to be sequestered from dietary ants. The cooccurrence in most frogs of related pumiliotoxin alkaloids **309A** and **325A** (see Fig. 1, Supplementary Information, and Table 1) suggests a possible common alkaloid-source-ant/symbiotic mite for these two alkaloids. Alternatively, *Mantella* could possibly have a hydroxylase to convert **309A** into **325A**, as evidence for frog hydroxylases has been suggested by alkaloid-feeding experiments with captive-born *Dendrobates* spp. (Daly et al., 2003) and *Pseudophryne* spp. (Smith et al., 2002). In one *Mantella* individual, SpiroP **236′** was considerably more abundant than **236** (see Supplementary Information chromatogram for frog #U4), similar to the relative abundance of these spiropyrrolizidines reported for the putative source millipede of Clark et al. (2005).

Frog Size and Prey Consumption

M. baroni in this study had an average SVL of 25.5 ± 2.2 mm (N = 15), with a range of 22–30 mm identical to a report by Vences et al. (1999) (Table 1). *M. bernhardi* SVLs ranged from 19 to 22 mm in Vences et al. (1999); however, the seven specimens in this study ranged from 17 to 22 mm with an average SVL of 19.9 ± 2.0 mm. *M. baroni* are significantly larger than *M. bernhardi* (*t*-test, $P < 0.001$), and based on the number of prey per preserved dissected stomach, the larger *Mantella* species consumes more prey (34.7 ± 14.8 prey/frog for 12 *M. baroni* vs. 16.6 ± 11.4 prey/frog for 7 *M. bernhardi*; *t*-test, P = 0.009, from Table 1 data). Furthermore, when performing a *regression* on one species within a locality, we find a linear relationship for *M. baroni* from Vohi, indicating that larger frogs consumed more prey (slope = 4.949, P = 0.031, R^2 = 0.832). A partial correlation for the 22 *Mantella*, controlled for by locality, also revealed that frog SVL is positively correlated with both the number of prey (correlation = 0.642, P = 0.004) and the total number of alkaloids per frog (correlation = 0.525, P = 0.025).

All dietary data represent a "snapshot" of what each specimen ate in the morning of its collection—frogs were preserved within 30 min of capture. In addition to consuming more, and thus probably a greater diversity of prey, it is also likely that some larger prey (that might contain unique alkaloids) are only consumed by larger frogs. Of the 609 *Mantella* stomach arthropods identified by Clark et al. (2005), the two largest prey items were recovered from *M. baroni* (data not shown).

Frog Alkaloid Diversity and Habitat Disturbance

The lower alkaloid diversity (average of 12.0 alkaloids/frog) at Ampa as compared to any other locality could merely be a reflection of the reduced arthropod consumption observed in this smaller species (Table 1, see above). An alternative explanation is suggested by the

notable link between frog alkaloid diversity and observed forest disturbance. The pristine primary forest of Vato is located within Ranomafana Park boundaries and contained *Mantella* with an average of 28.7 known alkaloids per frog (Table 1). Secondary roadside forest localities at the edges of Park boundaries yielded frogs with an intermediate diversity of skin alkaloids (average of 14.5 and 20.2 alkaloids/frog at Saha and at Vohi, respectively). The mean number of alkaloids per frog (mean frog alkaloid diversity) differed: (1) among the three localities of 1100-m elevation (ANOVA, $F = 6.304$, $P = 0.012$, $Eta^2 = 0.492$); (2) more significantly among all four localities (ANOVA, $F = 10.133$, $P < 0.001$, $Eta^2 = 0.628$); and (3) between the two elevation groups, whether (ANOVA, $F = 8.388$, $P = 0.009$, $Eta^2 = 0.295$, $n = 22$) or not (ANOVA, $F = 7.547$, $P = 0.013$, $Eta^2 = 0.284$, $n = 21$) the *M. madagascariensis* data point was included in the analysis. The Eta^2 value suggests that locality differences account for 62.8% of the observed variation in mean frog alkaloid diversity among the four localities.

Fragmented Ampa is clearly the most disturbed forest, with fragments surrounded by rice fields and other agriculture (VCC personal observation; also Rabemananjara et al., 2005). Only 20 different known alkaloids were detected in the six frogs from Ampa, as compared to more than 28 different alkaloids at each of the other localities; this is particularly striking since fewer frogs were sampled at most other localities (Table 1). Fragmentation could lower diversity of alkaloid-source arthropods by eliminating critical microhabitats. Invasive ants can also lower insect diversity in fragmented lowland forests (Fisher et al., 1998; Fisher, 2005). Although confounded by distance, variation in elevation, and frog size/species, this pattern suggests that future conservation research should aim to understand the impact of forest fragmentation on ant assemblages, as invasive ants might decrease the variety of alkaloid-source arthropods available to *Mantella* in Madagascar. Such a scenario could also be imagined for unrelated poison frogs of convergent ecosystems on other continents.

Temporal Variation in Alkaloid Content at Two Localities

Of the 30 alkaloids we detected in the January 1993 Saha 17-skin sample, 15 were absent from the 2003 Saha frogs and four of these were absent from all of the 2003 frog samples (Table 1). Similarly, on average 40% of alkaloids detected in each of the other three localities had been detected in the 1993 Saha sample; 1993 Saha shows little more resemblance to 2003 Saha (15/30 alkaloids) than to Vato (16/33), Vohi (22/59), or Ampa (7/20) frogs in 2003, and 1993 Saha even grouped with the Vohi frogs in the *K*-means cluster analysis (Table 1). In contrast, of the 27 alkaloids we detected in the December 1989 Vato 10-skin sample, only five were absent from Vato and three of these were absent from samples representing the entire region 14 yr later; 11 alkaloids were detected in 2003 Vato but not in 1989 Vato frogs. A comparison of 1989 Vato to different 2003 localities revealed that 1989 Vato shares more alkaloids (66%) with 2003 Vato than with any other locality—only about 33% of 1989—Vato alkaloids were detected in frogs from each of the other localities (17/59 at Vohi, 10/28 at Saha, and 7/20 at Ampa; see Table 1).

Alkaloids not reported in Daly et al. (1996) that are detected here include **151B**, **197E**, **217A**, **223H**, **233A**, **251N**, **251O**, **291E**, **293D**, **307G**, and **323E** in the 1993 Saha sample, and **151B**, **197B**, **251O**, **255B**, **257D**, **271B**, **275C**, **291E**, **293D**, **307A**, **309C**, **323E**, and **325A** in the 1989 Vato sample; these differences are likely a result of the greater sensitivity of the GC-TOF-MS used in this study as compared to the quadrupole GC-MS used in 1996.

Frogs retain their alkaloids for several years in captivity (Daly et al., 1997, 2003 and references therein), so it seems unlikely that the observed temporal variation was influenced

by seasonal variation in time of collection (December/January, the start of the rainy season, vs. March/April, the end of the rainy season). Rather, these results might indicate that the arthropod assemblage was relatively constant in the undisturbed primary forest of Vato, whereas disturbed roadside Saha had undergone a shift in the arthropod community. Disturbance and fragmentation of habitat could decrease the availability of alkaloid-containing prey and pose a possible threat to *Mantella* survivorship. This point is further supported by the observation that the two frog individuals with highest skin-alkaloid diversity both came from pristine Vato, and the six frog individuals with the lowest skin-alkaloid diversity were from disturbed roadside Saha and disturbed Ampa forest fragments (Table 1). A potential threshold diversity for alkaloid-containing arthropods required to maintain wild populations of poison frogs has not yet been determined.

Nonlethal Collection of Frog Skin Alkaloids with the TAS

There is no significant difference in the mean number of frog skin alkaloids detected using a nonlethal TAS vs. the traditional method (Daly et al., 1994b) of lethal full-skin extraction (*t*-test, $P > 0.05$, CI contains zero, for eight *M. baroni* of Vohi). The single TAS frog sample acquired at Saha contained more detectable known alkaloids than two skin samples and fewer alkaloids than one skin sample from Saha. However, quantitative detection by a flame-ionization GC-MS revealed that only 25% of the amount of each alkaloid is recovered with the TAS as compared to full-skin extraction (John W. Daly, personal communication). Based on our results, future investigations on frog alkaloid profiles should rely on the nonlethal TAS, especially when sampling endangered amphibians (listed on CITES appendices). The TAS is particularly useful for obtaining defensive compounds from frogs as it elicits a predator response in the frogs. The resulting TAS extract requires no further chemical workup, yet yields clean gas chromatograms consisting primarily of alkaloids.

Conclusions

Our data suggest that frog alkaloid profile variation is attributable to differences in arthropod assemblages among localities, and as such may give an indication of local ecosystem health. Specifically, variation in frog skin alkaloids appeared: (1) greatest among frog individuals of more geographically distant localities that varied in elevation and size of resident species; (2) lower among individuals from geographically close localities at the same elevation; and (3) lowest among *Mantella* individuals within a locality. In a comparison of frog alkaloid samples over a 10- or 14-yr period, alkaloid turnover, and thus presumably alkaloid-source arthropod turnover, was high in a disturbed locality and low in the pristine primary forest locality.

As all alkaloids in Malagasy poison frogs appear to be obtained from their diet, the local distribution of alkaloid-containing arthropods is likely key to variations in frog skin-alkaloid content among and within localities. Also, larger frog species consume more and larger prey and this increased arthropod sampling likely contributes to greater alkaloid diversity observed in frog skin. Further ecological studies on variation in poison frog alkaloids should use the TAS to evaluate in greater detail factors that might affect alkaloid diversity, such as habitat disturbance and elevation, frog gender, and especially frog age/size. Greater numbers of sympatric *Mantella* species should also be analyzed for variation in their defensive skin chemistry; only one *M. madagascariensis* was compared to several sympatric *M. baroni* (Clark et al., 2005; current study).

It appears that the alkaloid content of Malagasy poison frogs is most affected by locality, and that poison frogs continue to accumulate alkaloids as they age. This poison collection may reach some maximum diversity dependent on the assemblage of alkaloid-source species in the frogs' local habitat and/or individual territories. Because the alkaloids sequestered by frogs vary considerably in toxicity, it is to the frogs' advantage to acquire as many different defensive chemicals as possible. Accordingly, frogs living in particularly arthropod-rich habitats could obtain more dietary alkaloids.

Habitat quality and local arthropod diversity appear to affect how well poison frogs are chemically defended. Therefore, habitat disturbance and fragmentation might decrease *Mantella* survivorship or even exclude the poison frogs altogether *via* selective pressures. Future studies need to address this relationship and determine the threshold of alkaloid-containing arthropods required in habitat suitable for *Mantella* poison frogs.

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