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Individual and Geographic Variation of Skin Alkaloids in Three Species of Madagascan Poison Frogs (*Mantella*)

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Abstract Alkaloid profiles for 81 individual mantellid frogs, *Mantella baroni* (Boulenger 1988) (*N*=19), *M. bernhardi* (*N*=51), and *M. madagascariensis* (Grandidier 1877) (*N*=11), from six different populations from Madagascar were examined. Marked individual differences in alkaloid composition (number, type, and amount) were observed between different species and between populations of the same species. Disjunct populations of each of the three species differed significantly in alkaloid composition. Sympatric populations of *M. baroni* and *M. madagascariensis* also differed significantly in alkaloid composition. In *M. bernhardi*, differences in alkaloid composition were marginally associated with different sexes. A total of 111 alkaloids, including isomers, were detected in analysis of the individuals from the three species. The majority (47%) appear likely to be obtained from dietary mites, whereas many of the others (18%) are presumed to be from ants, and a few (4%) are from millipedes. Putative dietary sources for the remaining alkaloids are generally unknown, but beetles are probably the source of at least some of the tricyclic alkaloids (6%). In addition, alkaloid compositions from extracts of groups of individuals from five additional populations of *M. baroni* and from one population of *M. bernhardi* (Vences et al. 1994) and one population of *M. cowanii* (Boulenger 1882) were examined. An additional 50 alkaloids, including isomers, were detected in the combined samples, bringing the total number of alkaloids identified from these four species of mantellid frogs to 161. Alkaloid compositions in mantellid poison frogs are diverse and highly dependent on geographic location that appear to be largely determined by the nature and availability of alkaloid-containing prey items.

Keywords Alkaloids · Ants · Chemical defense · Chemical sequestration · Mantellid frogs · Mites · Trophic relationships · Vertebrate

Abbreviations

ANOSIM	analysis of similarity
FAME	fatty acid methyl ester
GC–MS	gas chromatography–mass spectrometry
3,5-I; 5,8-I	disubstituted indolizidine
5,6,8-I	trisubstituted indolizidine
nMDS	nonmetric multidimensional scaling
PTX	pumiliotoxin
aPTX	allopumiliotoxin
hPTX	homopumiliotoxin

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3,5-P	3,5-disubstituted pyrrolizidine
1,4-Q	1,4-disubstituted quinolizidine
SVL	snout-to-vent length
Spiro	spiropyrrolizidine
Tri	tricyclic alkaloid
ZCMV	zoological collection Miguel Vences

Introduction

The wide variety of lipophilic alkaloids present in skin extracts of poison frogs of the Neotropics (Dendrobatidae), subtropical South America (Bufonidae, *Melanophryniscus*), and Madagascar (Mantellidae, *Mantella*) appears to be directly sequestered from dietary arthropods (Daly et al. 1994a, b, 1997; Daly 1998). The putative arthropod sources of such alkaloids are as follows: (1) The widespread pumiliotoxins (PTXs) appear to be derived from oribatid mites (Takada et al. 2005; Saporito et al. 2006, 2007a), although two such PTX alkaloids have been reported from Panamanian formicine ants (Saporito et al. 2004). (2) The several classes of izidines with branch points in their carbon skeleton also appear likely to be derived from oribatid mites (Takada et al. 2005; Saporito et al. 2006, 2007a). However, one such izidine, a 5,8-disubstituted indolizidine, has been reported from a Madagascan myrmicine ant (Clark et al. 2005). (3) The izidines without branch points in their carbon skeleton appear to be derived from myrmicine ants, as are (4) the unbranched pyrrolidines and piperidines (Jones et al. 1999). Recently, two unbranched 3,5-disubstituted pyrrolizidines were reported from a formicine ant, and a different 3,5-disubstituted pyrrolizidine was reported from a ponerine ant (Clark et al. 2006). A 2,5-disubstituted pyrrolidine also was reported from the same species of formicine ant (Clark et al. 2006). In addition, one unbranched 3,5-disubstituted indolizidine and two unbranched pyrrolidines have been detected in oribatid mites (Saporito et al. 2007a). It seems likely that the ultimate source of alkaloids with branching in the carbon skeletons will be oribatid mites and the source of those with unbranched structures will be myrmicine ants (Saporito et al. 2007a). (5) Spiropyrrolizidine alkaloids appear to be derived from siphonotid millipedes (Saporito et al. 2003; Clark et al. 2005); however, one such alkaloid was recently reported from an oribatid mite (Saporito et al. 2007a). (6) Tricyclic alkaloids, such as precoccinelline, appear to be derived from coccinellid beetles (Daloze et al. 1995); however, precoccinelline and other tricyclic alkaloids were also recently reported from oribatid mites (Takada et al. 2005; Saporito et al. 2007a).

Variation in alkaloid composition (the number, type, and amount) within and among species has been reported for Neotropical dendrobatid poison frogs (Myers and Daly 1976; Daly et al. 1987, 1992, 2000, 2002; Myers et al. 1995; Saporito et al. 2006, 2007b), bufonid poison frogs (Garraffo et al. 1993a; Mebs et al. 2005; Daly et al. 2007), and mantellid poison frogs (Garraffo et al. 1993b; Daly et al. 1996; Clark et al. 2005, 2006). Individual variability in alkaloid composition has been reported for dendrobatids (Daly et al. 1994a; Myers et al. 1995; Saporito et al. 2006), bufonids (Mebs et al. 2005; Daly et al. 2007), and mantellids (Clark et al. 2006). The literature indicates that alkaloid compositions are strongly dependent on geographic location and that compositions change with time. Differences in habitat among locations and changes in habitat over time (succession) are likely responsible for determining the availability of alkaloid-containing prey arthropods, which is reflected as geographic and temporal variation in alkaloid composition of poison frogs (Daly et al. 1987, 1996, 2002; Saporito et al. 2006, 2007b).

This study was designed to provide further insight into the factors that are involved in alkaloid composition variability within and among populations of poison frogs of the mantellid genus *Mantella*. The poison frogs of this genus consist currently of 16 described and, probably, at least one undescribed species that are found in a variety of habitats in Madagascar (Vences et al. 1999; Glaw and Vences 2006). All of these species are small, diurnal frogs that have aposematic coloration, practice microphagy, and accumulate dietary alkaloids that act as a defense against predators (Daly et al. 1996, 1997; Vences and Kniel 1998; Vences et al. 1998; Schaefer et al. 2002). Alkaloid compositions have been reported for 11 of the species (see Garraffo et al. 1993a; Daly et al. 1996; Clark et al. 2005, 2006). In this paper, we report our findings of individual alkaloid composition for 81 individuals, comprising six populations of three different species of *Mantella* from Madagascar (*Mantella baroni* of the *M. cowanii* group, *M. madagascariensis* of the *M. madagascariensis* group, and *M. bernhardi*, the sole member of the *M. bernhardi* group [Pintak et al. 1998; Vences et al. 1999, 2004]). Individuals were sampled from two sympatric populations of *M. baroni* and *M. madagascariensis* and from two geographically distant populations of *M. bernhardi*. Profiles of the major, minor, and trace alkaloids are presented for the 81 individuals. In addition, we also report on a number of extracts from groups of *M. baroni*, *M. bernhardi*, and *M. cowanii*. Alkaloid composition for mantellid frogs was examined for possible relationships with geographic location (habitat), species, size, and sex. Geographic location (and associated habitat) appears to be the primary determinant of variation in alkaloid composition; however, differences among sympatric species were also observed.

Methods and Materials

Mantellid Frog Collections—Individual Frog Analyses A total of 81 mantellid frogs of three species were collected: *M. baroni* (*N*=19), *M. bernhardi* (*N*=51), and *M. madagascariensis* (*N*=11). Each of the three species was collected at two different locations (Fig. 1), and therefore, a total of six different populations were examined. All frogs sampled from one site were usually collected during the same day, sometimes during a time span of 2–3 days, by opportunistic searching that involved observation and removal of leaf litter and low vegetation or by precisely targeting calling males, especially in the case of *M. baroni*. All frogs were collected on relatively small plots of a maximum of 0.5 ha, often much smaller. For instance, all *M. bernhardi* from Vevembe were found within an area of 20×20 m and all *M. madagascariensis* from Ranomafana within an area of 10×10 m, in a small degraded area under *Eucalyptus* trees near primary rainforest.

Individual frogs were sexed and measured for snout-to-vent length (SVL) to the nearest 0.1 mm. Collection localities included Ranomafana, a relatively large National Park in southeastern Madagascar, which contained several specific collecting localities for *Mantella* (e.g., Ranomafanakely, Mangevo, Vatoharanana, Vohiparara; Fig. 1). Individual skin extracts reported herein to originate from Ranomafana all came from a specific collection site, locally known as "Ranomafanakely," along National Road 45 from the village of Vohiparara toward the town of Fianarantsoa. The combined skin extracts reported herein to originate from Vohiparara came from the Kidonavo Bridge. Besariaka is located far to the north of Ranomafana, whereas Manombo and Vevembe are far to the south (Fig. 1). At two sites, *M. baroni* and *M. madagascariensis* were collected in syntopy. At Ranomafana, *M. madagascariensis* was found close to the road in a tiny patch of degraded forest dominated by *Eucalyptus* spp., whereas *M. baroni* was found at a distance of less than 50 m in primary rainforest. At Besariaka, the two species were fully mixed and, apparently, were using exactly the same microhabitat.

Collection dates and the total number of frogs sampled at each location are provided in table headings. The global positioning system (GPS) coordinates and nature of each site and voucher identification numbers are tabulated in the Supplementary Information. Voucher specimens are deposited at the Zoological Museum Amsterdam, the Université d'Antananarivo, Département de Biologie Animale (UADBA), and the Zoologische Staatssammlung München.

Mantellid Frog Collections—Combined Frog Analyses In January–February, 2003, 29 mantellid frogs from three species, *M. baroni* (*N*=19), *M. bernhardi* (*N*=8), and *M.*

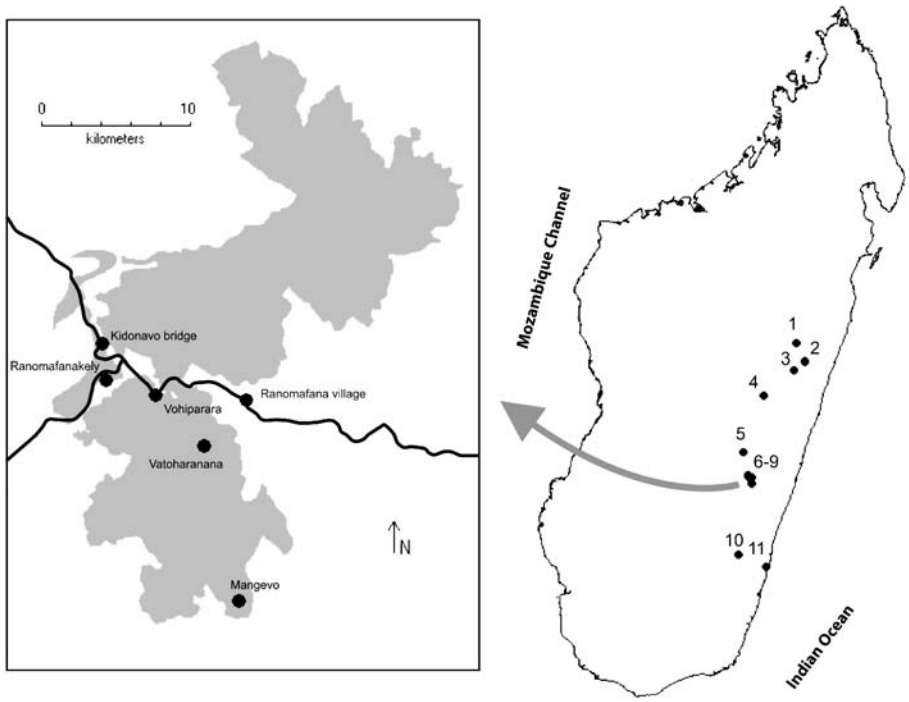

Fig. 1 Map of localities in Madagascar where *Mantella* frogs were collected including Andriabe (*1*), Vohindrazana (*2*), Besariaka (*3*), Tsinjoarivo (*4*), Antoetra (5), Vatoharanana (*6*), Vohiparara (*7*), Mangevo (*8*), Ranomafanakely (*9*), Vevembe (*10*), and Manombo (*11*). The *map on the left* shows detailed geographical location of localities 6–9. Ranomafana National Park is indicated in *gray*

cowanii (*N*=2), were also collected for extracting alkaloids from samples of pooled individuals. *M. baroni* was collected from five different populations, whereas *M. bernhardi* and *M. cowanii* were collected from one population each (Fig. 1). Individual frogs of the same species from each site were combined for alkaloid analyses, and therefore, alkaloid composition is based on combined skin samples. Collection dates and the total number of frogs sampled at each location are provided in table headings. The GPS coordinates and nature of each site are tabulated in the Supplementary Information. Voucher specimens are deposited as described above for the frogs that were extracted individually.

Spectral Analyses A Finnigan–Thermoelectron gas chromatography–mass spectrometry (GC–MS) (GCQ) was used to obtain all mass spectral data reported here. The GC was fitted with a Restek-5MS (Bellefonte, PA, USA) fused silica column (30 m×0.25 mm inside diameter [i.d.], 0.25-μm film thickness) and used a temperature program of 100 to 280°C at 10° per min with a final hold time of 5 min. The injector temperature was 280°C. The carrier (He) flow was controlled at 1 ml/min. The gas chromatography–Fourier transform infrared spectrometry (GC-FTIR) spectral analyses were obtained with an HP-5 (Hewlett-Packard, USA) fused silica-bonded capillary column (25 m× 0.32 mm i.d., ×0.17 μm film thickness) programmed from 100 to 280° at a rate of 10° per min, interfaced with an HP model 5971 Mass Selective Detector and an HP Model 5965B IRD detector (narrow band 4,000–750 cm^{-1}). An HP ChemStation was used to generate MS and FTIR spectra. For additional details of spectral analyses, see Saporito et al. 2006 and 2007b.

Individual Frog Skin Analyses Individual mantellids were examined for alkaloids by using GC–MS. In many cases, chemical ionization-mass spectrometry (ND_3) was used to confirm molecular ions and the number of exchangeable hydrogens. Alkaloids were identified by comparison of spectral and chromatographic properties to previously detected and identified poison frog alkaloids (see Supporting Information of Daly et al. 2005 for a complete listing). The single skin samples were stored in small plastic vials sealed with a silicone rubber O-ring with approximately 0.5–1.5 ml of methanol, which unfortunately led to contamination of each sample with a series of silicone polymers and dibutyl phthalate. Because of the large number of samples examined in this study and the length of time necessary for complete alkaloid partitioning (see below for combined skin analyses), we developed a fairly rapid "semi-purification" of the samples for GC–MS analysis. The method is as follows: To 50 μl of the methanolic frog skin extract, 50 μl of a 0.1 M solution of HCl in methanol was added and swirled well. Then, the methanol was immediately blown off with a stream of nitrogen gas. The HCl in methanol had been prepared by addition of acetyl chloride to methanol, to form a 1 M solution, which was then diluted 1:10 with additional methanol. After evaporation, the residue consisted of hydrochlorides of any frog skin alkaloid and nonvolatile contaminants. It was redissolved in a small volume of methanol, followed by re-evaporation. This process was repeated twice. Then, 50 μl of reagent grade octane was added. The octane was blown off with nitrogen to remove any residual methanol. Then, a small portion of octane was added, swirled with the sample residue, and removed with a pipette, thereby removing the neutral silicones, phthalates, and fatty acid methyl esters (FAMEs). This was repeated twice, and the final traces of octane were removed with a nitrogen stream leaving a whitish residue of amine hydrochlorides. The residue was dissolved in 50 μl of methanol and the vial tightly capped with a Teflon-lined APC (Alltech, Deerfield, IL, USA) cap for later alkaloid analysis. One microliter was injected for the GC–MS analysis (see above). The octane washes were occasionally combined from a group of samples and checked for any dissolved amine hydrochlorides. Traces of the lower molecular weight amine hydrochlorides were detected in some extracts, but the large majority of materials were silicone polymers, dibutyl phthalate, and FAMEs. Each sample could be prepared in less than 10 min. Only one sample was processed at a time to ensure minimum contact between the methanolic HCl and alkaloid mixtures to avoid any acid-catalyzed reactions. We cannot rule out the possibility that some GC peaks represent acid-catalyzed artifacts. To check for possible artifacts, aliquots from each set of individual methanolic skin extracts were combined, and an alkaloid fraction was obtained by the standard partitioning under mild conditions and analyzed (see below). The volumes of the skin extracts varied by a factor of two to three, and no attempt was made to apply a correction, as the weights of frog skin, while probably fairly uniform, were unknown. Thus, quantitation is an approximation, but there were large differences in the overall amounts, ranging in MS total ion currents by 10^2 (see legends to tables). Some samples had no detectable or barely detectable levels of alkaloids. Combined samples consisting of aliquots from all individuals of the same species and collection sites were also subjected to alkaloid partitioning. For example, 50 μl of each individual extract of *M. baroni* from the Ranomafana site was combined for standard alkaloid-partitioning as previously described (Daly et al. 1994a). Such combined samples, much more concentrated than the single skin samples, were used to help establish retention times and alkaloid compositions for the single skin samples as well as an aid in ruling out any

acid-catalyzed reactions potentially occurring during the individual skin protocols. Such artifacts, not detected in the partitioned alkaloid fraction, proved to be rare and minor and are not reported.

Despite the individual skin protocols having problems of not excluding completely neutrals like silicones, phthalates, and FAMEs and possibly generating artifacts arising from the inadvertent acid catalysis of unwanted reactions with methanol, there may be an advantage in retaining very volatile alkaloids by virtue of having converted them to hydrochlorides. Some extracts (no. 113 of Table 3) had low molecular weight alkaloids (e.g., **197D**, **199B**), not observed after using the standard partitioning protocol.

Combined Frog Skin Analyses An alkaloid fraction was prepared from the methanol extract of the combined skins by using the partitioning methodology as described in Daly et al. (1994a, b). The resultant alkaloid fractions were analyzed spectrally by GC–MS and, in some cases, by GC-FTIR [for details of the GC–MS and GC-FTIR spectral analysis, see Saporito et al. (2006, 2007b)].

Statistical Analyses—Individual Frogs Variation in alkaloid composition within and among mantellid populations was visualized graphically by using nonmetric multidimensional scaling (nMDS). In nMDS plots, individuals/populations that have greater similarity in alkaloid compositions will be plotted closer to each other than individuals/populations with very different alkaloid compositions [see Saporito et al. (2006, 2007b) for further examples and discussions on the use of these techniques]. Differences in alkaloid composition among these populations were analyzed with a one-way analysis of similarity (ANOSIM). Alkaloid composition is a simultaneous measure of the number, type, and amount of alkaloids, and therefore, the use of nMDS plots in association with ANOSIM provides a more biologically meaningful view of alkaloid variation in poison frogs as compared to individual analyses of the number and amount of alkaloids. Differences in alkaloid composition between sexes for *M. bernhardi* from Mandombo were also visualized by using nMDS, and differences were analyzed with a one-way ANOSIM. All nMDS plots and ANOSIM results are based on Bray–Curtis dissimilarity matrices. All nMDS and ANOSIM statistical analyses were performed by using the software program PRIMER (version 5; Clarke and Warwick 2001).

Linear regression was used to determine if the total number of alkaloids (a measure of alkaloid diversity) varied with size of the frog (measured as SVL) within and among the three different mantellid species. The statistical program SPSS (version 11.5 for Microsoft Windows) was used to perform these statistical analyses.

Results

A total of 111 alkaloids, including isomers, were identified from skin extracts of individuals of *M. baroni* (*N*=19; Tables 1 and 2), *M. bernhardi* (*N*=51; Tables 5 and 6), and *M. madagascariensis* (*N*=11; Tables 3 and 4). Representatives of all classes of alkaloids noted in "Introduction" were present in at least one species or population (Table 7). Twenty representative alkaloids were detected relatively frequently in this study [Fig. 2, see Daly et al. (2005) for details concerning structures of the more than 800 alkaloids reported to date from alkaloid-containing amphibians]. A total of 82 alkaloids, including isomers, were detected in skin extracts of the combined samples (ranging from one to eight skins per sample) of *M. baroni*, *M. bernhardi*, and *M. cowanii* (Tables 8, 9, 10, and 11). Representatives of all classes of alkaloids noted in "Introduction" were present in at least one species or population (Table 11). Fifty alkaloids that were detected in these combined frog samples, mainly 5,8-disubstituted indolizidines and alkaloids of undefined structure (unclassified alkaloids), were not detected in any of the individual frog samples (cf. Tables 7 and 11). Thus, 161 alkaloids were detected in this study of four mantellid species (Tables 7 and 11).

Previously unreported new alkaloids are indicated by asterisks within the text and tables. Their GC retention times, mass spectral data, and other data are presented in the Supplementary Information. Tentative structures for some of these previously unreported new alkaloids are proposed in the Supplementary Information.

Individual Skin Alkaloid Analyses—M. baroni from Ranomafana Many of the alkaloids identified in 15 skin extracts of *M. baroni* from Ranomafana were of the PTX group (Table 1). The dominant PTX alkaloids in most of the extracts were PTX **251D** and **309A** and homoPTX **265N**. Other alkaloids of the homoPTX class (**251R**, **281K**) were detected in trace amounts. Only one alloPTX was detected (**325A**), which also usually occurred in trace amounts. Several extracts had trace or minor levels of PTX **237A** (a C-15 analog of the C-16 PTX **251D**). A keto-PTX **307F′** (characterized by an enhanced *m*/*z* 194 ion) was detected in most of the extracts, but only as a trace or minor alkaloid. PTX **267C** and deoxyPTX **251H** occurred in skin extracts rarely and usually as trace alkaloids. In addition to alkaloids of the PTX group, many individuals of *M. baroni* from Ranomafana contained significant amounts of 1,4-disubstituted quinolizidines **217A** and **231A**, along with 5,8-disubstituted indolizidine **217B**. The 5,6,8-trisubstituted indolizidine **273A** was present in large amounts in nearly every extract, often accompanied by a minor diastereomer. The PTX group of alkaloids (PTX, alloPTX, and homoPTX) and the branched chain indolizidine and

Table 1 Alkaloid profiles for *Mantella baroni* (15 individuals) from Ranomafana, 22 January 2004

ZCMV# + sex[a]	SVL (mm)	Amount[b]	Pumiliotoxins/homopumiliotoxins						
			Mites						
			237A (PTX)	**251D** (PTX)	**251H** (deoxyPTX)	**251R** (hPTX)	**265N** (hPTX)	**267C** (PTX)	**281K** (hPTX)
126f	27.5	+++	2	3		1	3		
127m	25.0	+++	1	2		1	3		1
128s	20.4	+		1				1	
129m	24.7	+++	1	2			3		
130m	23.6	++		2			2		
131m	23.6	+++	1	2		1	2		
132m	21.7	+++	1	2			2		
133f	27.1	+++	*1*	3		1	3/1		
134m	24.7	+++	1	2			2		
135m	22.3	++	2	2			3		
136m	22.7	+++	1	3		1	2		
137m	24.6	+++	1	3	1		2	1	
138m	23	+++	1	3		1	3		
139m	23	++		3			3		
140m	23.5	+++	1	2			3		

ZCMV# + sex[a]	Pumiliotoxins/homopumiliotoxins				Izidines				
	Mites								
	293D (deoxyPTX)	**307F′** (PTX)	**309A** (PTX)	**325A** (aPTX)	**205L**[c] (dehydro -5,8-I)	**217A** (1,4-Q)	**217B** (5,8-I)	**231A** (1,4-Q)	**233A** (1,4-Q)
126f		2	2			1	1	1	1
127m		2	3	1		2	2	2	1
128s									
129m		1	2				2	3	1
130m		1	2			2	1	1	
131m		1	3			2/1	1	1	
132m		1	3			2/2	1	2	
133f			3	1		2	2	3	
134m		1	3			1	1	2	
135m		1	3		1	2	2	3/1	
136m		2	2			2	1	2	
137m	1	2	2	1		1	1		1
138m			2	1	1	1	1	1	
139m		1	2			3		2	
140m			1	1	1	1	2	2	

ZCMV# + sex[a]	Izidines							Spiros/tricyclics/unclass
	Mites			Ants				Millipedes/Beetles/Unknown
	251N (5,8-I)	**273A** (5,6,8-I)	**293O**[c] (dehydro-5,8-I)	**249A** (3,5-I)	**249I** (3,5-P)	**251O** (3,5-P)	**275C** (3,5-I)	Other
126f		2/1		1	1		/2/	
127m		2/1					/2/2	
128s								
129m		2/1				2/1	/2/	
130m		1		1			1/3/	
131m		2/1				1	/1/2	
132m		2/2					/2/	Spiro **236** (1)

Table 1 (continued)

ZCMV# + sex[a]	Izidines							Spiros/tricyclics/unclass
	Mites			Ants				Millipedes/Beetles/Unknown
	251N (5,8-I)	**273A** (5,6,8-I)	**293O**[c] (dehydro-5,8-I)	**249A** (3,5-I)	**249I** (3,5-P)	**251O** (3,5-P)	**275C** (3,5-I)	Other
133f		2/1		1			/2/2	
134m		2					/1/	Spiro **236** (1)
135m	1	2					/2/	Spiro **236** (1)
136m		2					/2/2	
137m	1	2/1					1//1	Spiro **236** (1)
138m		2/1	1	1		1	1/2/	Spiro **236** (1); Unclass **207N** (1)
139m		2					//2	
140m		3/2				2	//2	Spiro **236** (1)

Probable class and dietary source of each alkaloid are indicated in the headings (see abbreviations).
[a] Sex (*m* male; *f* female, *s* subadult) is indicated.
[b] Total content of alkaloids [major (+++), minor (++), trace (+)] are based upon total ion chromatogram intensities with 10^4 or greater = major, 10^3–10^4 = minor; $\leq 10^3$ = trace. The amounts of each alkaloid in the table are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two or three intensities are tabulated, two or three isomers are noted, and the intensities are in the order of elution from the GC column. Blanks indicate the alkaloid was not detected.
[c] Alkaloids reported for the first time. See Supplementary Information for characterization.

quinolizidine alkaloids of *M. baroni* from Ranomafana are likely derived from oribatid mites (Saporito et al. 2007a). Izidines with unbranched carbon skeletons, which likely are sequestered from myrmicine ants (Jones et al. 1999), were rare in *M. baroni* from Ranomafana. The only exception was the presence of diastereomers of the 3,5-disubstituted indolizidine **275C**, which were present in all but one individual and often in large amounts. The spiropyrrolizidine **236** of millipede origin (Saporito et al. 2003; Clark et al. 2005) occurred in six of the 15 individuals of *M. baroni* from Ranomafana.

Individual Skin Alkaloid Analyses—M. baroni from Besariaka The skin extracts of four *M. baroni* from Besariaka (all adult males; Table 2) differed from the same species collected from Ranomafana (Table 1). However, alkaloid composition among individuals was again characterized by many alkaloids of the PTX group, including PTXs **251D**, **307F′**, **307G**, and **309A**, which were found in all frogs. No homoPTXs were detected. Two alloPTXs (**323J*** and **325A**) were identified in large amounts in most frogs. Keto-PTXs **307F″** and **307F‴** (characterized by an enhanced *m*/*z* 193 fragment ion) occurred in one extract along with the more common **307F′**. Similar to the *M. baroni* from Ranomafana, frogs from Besariaka contained large amounts of 1,4-disubstituted quinolizidine **217A**. Frogs from Besariaka had a somewhat greater diversity of putative mite izidine alkaloids (*N*=13), as compared to frogs from Ranomafana (*N*=11). The 5,6,8-trisubstituted indolizidine **223A** and dehydro-5,8-disubstituted indolizidine **245F**, both of which are putative mite alkaloids, occurred in three of the four Besariaka frogs. Neither of these two alkaloids was detected in Ranomafana frogs. Putative ant alkaloids in the Besariaka frogs were represented by trace amounts of the 3,5-disubstituted pyrrolizidine **223M** in one frog and by the 3,5-disubstituted indolizidine **251O** in two frogs. Interestingly, the 3,5-disubstituted indolizidine **275C**, common in *M. baroni* from Ranomafana, was not detected in *M. baroni* from Besariaka.

Alkaloid Variation Within and Among Populations of M. baroni As previously documented for *M. baroni* (Daly et al. 1996; Clark et al. 2006), alkaloid composition within and among populations of this species can differ markedly (see Tables 1 and 2 for extracts of individuals from two populations and Tables 8 and 9 for extracts of groups from five populations). Alkaloid compositions among the 15 individual frogs from Ranomafana were more similar to each other than were the compositions of the four individual frogs from Besariaka (see nMDS plot of Fig. 3a). Alkaloid composition of *M. baroni* was significantly different between Ranomafana and Besariaka (Global R=0.99; P<0.001; Fig. 3a).

Individual Skin Alkaloid Analyses—M. madagascariensis from Ranomafana Alkaloid composition of six extracts of *M. madagascariensis* from Ranomafana (Table 3) differed from that of *M. baroni* from Ranomafana (Table 1). However, it should be noted that the two species were not in exactly the same microhabitat at the Ranomafana site (see "Methods and Materials"). Most individuals of *M.*

Table 2 Alkaloid profiles for *Mantella baroni* (four individuals) from Besariaka, 15 February 2004

ZCMV[#] + sex[a]	SVL (mm)	Amount[b]	Pumiliotoxins							
			Mites							
			251D (PTX)	**265X** (deoxyPTX)	**267C** ((PTX)	**281N** (deoxyPTX)	**293D** (deoxyPTX)	**307F′** (PTX)	**307F″/F‴** (PTX)	**307G** (PTX)
911 m	24.9	+++	1					1		1
912 m	27.1	+++	3					1		2
913 m	26.2	+++	1					2		1
914 m	26.0	+++	1	1	1	1	1	1	1/1	1

ZCMV[#] + sex[a]	Pumiliotoxins			Izidines						
	Mites									
	309A (PTX)	**323J**[c] (aPTX)	**325A** (aPTX)	**217A** (1,4-Q)	**223A** (5,6,8-I)	**231A** (1,4-Q)	**233A** (1,4-Q)	**245F** (dehydro5,8-I)	**247E** (5,8-I)	**249BB**[c] (5,6,8-I)
911 m	2	2	3	3	1	2		1	1	
912 m	2	2	3	2	1			2		1
913 m	2	2	3	2	2		1			
914 m	1/2		1/2	3/1		1		1	1	

ZCMV[#] + sex[a]	Izidines							Spiros/tricyclics/unclass
	Mites					Ants		Millipedes/beetles/unknown
	251N (5,8-I)	**265F** (dehydro5,8-I)	**265U** (5,6, 8-I)	**267E** (5,8-I)	**267S** (5,8-I)	**223M** (3,5-P)	**251O** (3,5-I)	Other
911 m		1						Tri **245J** (2); Unclass **249AA**[c] (1)
912 m	1	1	1		1	1	1/1	
913 m							1	Tri **245J** (1)
914 m		1		1	1			Tri **245J** (2); Unclass **307L**[c] (1)

The probable class and dietary source of the alkaloid are indicated in the heading (see abbreviations).
[a] Sex (*m* male) is indicated.
[b] Total content of alkaloids [major (+++)] is based upon total ion chromatogram intensities with 10^4 or greater. The amounts of each alkaloid are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two intensities are tabulated, two isomers are noted, and the intensities are in the order of elution from the GC column. Blanks indicate the alkaloid is not detected.
[c] Alkaloids reported for the first time. See Supplementary Information for characterization.

madagascariensis had substantial amounts of homoPTX **265N**, as was the case for the *M. baroni*. However, in three of the six *M. madagascariensis*, homoPTX **281K** was present as a dominant or substantial alkaloid, whereas the same alkaloid was present in only one of the 15 *M. baroni* from Ranomafana and then only as a trace alkaloid. PTXs **237A** and **309A**, abundant in *M. baroni*, occurred only as trace alkaloids in *M. madagascariensis*. PTX **251D**, a dominant or substantial alkaloid in all but one of the 15 *M. baroni*, occurred in substantial amounts in only one of the six *M. madagascariensis* while being a trace alkaloid in another four. PTX **267C**, which was detected only twice as a trace alkaloid in 15 *M. baroni*, occurred in three of the six *M. madagascariensis*, once as a major alkaloid, once as a minor alkaloid, and once as a trace alkaloid. There were considerable differences between *M. madagascariensis* and *M. baroni* in the izidine alkaloids that are of putative mite origin. The 1,4-disubstituted quinolizidines **217A** and **231A**, common in large amounts in *M. baroni*, were less common in *M. madagascariensis*. Similarly, the 5,8-disubstituted indolizidine **217B** was much less common in *M. madagascariensis*. The six *M. madagascariensis* frogs contained eight 3,5-disubstituted pyrrolizidines and indolizidines, which are of probable ant origin. This is in marked

Table 3 Alkaloid profiles for *Mantella madagascariensis* (six individuals) from Ranomafana, 22 January 2004

ZCMV# + sex[a]	SVL (mm)	Amount[b]	Pumiliotoxins/homopumiliotoxins								
			Mites								
			237A (PTX)	**251D** (PTX)	**251H** (deoxyPTX)	**251R** (hPTX)	**265N** (hPTX)	**267C** (PTX)	**281K** (hPTX)	**309A** (PTX)	**323A** (PTX)
109f	22.2	+++		2	1		3	3	3/1	1	1
110m	18.2	+++		1			3		2		1
111m	19.4	+		1			2	1	1	1	
112m	19.2	n.d.									
113m	20.6	+++		1							
114f	22.4	+++	1	1		1	3	2	3/1	1	

ZCMV# + sex[a]	Izidines										
	Mites										
	203A (5,8-I)	**207W**[c] (dehydro5,8-I)	**217A** (1,4-Q)	**217B** (5,8-I)	**231A** (1,4-Q)	**251N** (5,8-I)	**267S** (5,8-I)	**273A** (5,6,8-I)	**289G**[c] (5,6,8-I)	**293O**[c] (dehydro5,8-I)	**309K**[c] (izidine)
109f	1		3	1	1		1	3	1		
110m	1		3		1	1	1	1	1		
111m					1			1			
112m											
113m		1			1			1		1	1
114f	1		2					2/2	1		

ZCMV# + sex[a]	Izidines									Spiros/tricyclics/unclass
	Ants									Millipedes/beetles/unknown
	197J[c] (3,5-P)	**239K** (3,5-P)	**249A** (3,5-I)	**251O** (3,5-P)	**263S**[c] (3,5-P)	**265W** (3,5-P)	**267H** (3,5-P)	**275C** (3,5-I)	**291J** (izidine)	Other
109f	1	1	2		1			2	1	Spiro **236** (2/1); Tri **263T**[c] (1);
110m				1				1/1		Spiro **222** (1), **236** (2/1); Tri **247N**[c] (1), **263M** (1)
111m								1		Spiro **236** (1)
112m										
113m			1	1			3	1/1		Spiro **222** (1), **236** (3/1), **252B** (1); unclass **183C** (1),**197D**(1), **199B**[c] (1),
114f				1		1	2	1	1	Spiro **236** (2/1)

The probable class and dietary source of the alkaloid are indicated by the headings.

[a] Sex (*m* male; *f* female) is indicated.

[b] Total content of alkaloids [major (+++), trace (+)] is based upon total ion chromatogram intensities with 10^4 or greater = major, $\leq 10^3$ = trace; n. d. = none detected. The amounts of each alkaloid are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two intensities are tabulated, two isomers are noted, and the intensities are in the order of elution from the GC column. Blanks indicate the alkaloid is not detected.

[c] Alkaloids reported for the first time. See Supplementary Information for characterization.

contrast to the 15 *M. baroni* frogs, where only four such alkaloids were detected. Both species had the presumed ant-derived 3,5-disubstituted indolizidine **275C** as a common constituent. In addition, the millipede alkaloid **236** was identified in 6 of the 15 *M. baroni* and in 5 of the 6 *M. madagascariensis*. Interestingly, in *M. madagascariensis*, a minor isomer of the spiropyrrolizidine **236** with a slightly longer retention time occurred in four of the frogs. Unfortunately, a GC-FTIR spectrum of this minor isomer could not be obtained. A minor isomer of **236** has been previously reported from *M. baroni* (Clark et al. 2005) and has been found to occur in siphonotid millipedes

Table 4 Alkaloid profiles for *Mantella madagascariensis* (five individuals) from Besariaka, 15 February 2004

ZCMV# + sex[a]	SVL (mm)	Amount[b]	Pumiliotoxins							Izidines
			Mites							
			251D (PTX)	**305A** (aPTX)	**305C** (aPTX)	**309A** (PTX)	**321C** (aPTX)	**323B** (aPTX)	**325A** (aPTX)	**211B** (izidine)
915 f	23.6	+++	1	1	1	1	1	1	1	
916 m	20.9	+++		1		1	2	1	1	1
917 f	25.0	+++		1			2	1/1		
918 m	21.8	+						1	1	
919 f	24.9	+						1	1	

ZCMV# + sex[a]	Izidines								
	Mites							Ants	
	217A (1,4-Q)	**223A** (5,6,8-I)	**239C** (5,6,8-I)	**239Z**[c] (5,6,8-I)	**267E** (5,8-I)	**267S** 5,8-I)	**269J**[c] (5,6,8-I)	**195B** 3,5-I)	**211E** (3,5-I)
915 f	1	1	1	3/1	1	1		1	
916 m	1	1	1	3					1
917 f	1	1	1	3/1			1		
918 m	1			1					
919 f	1			1					

The probable class and dietary source of the alkaloid are indicated in the headings (see abbreviations).
[a] Sex (*m* male; *f* female) is indicated.
[b] Total content of alkaloids [major (+++), trace (+)] is based upon total ion chromatogram intensities with 10^4 or greater = major; $\leq 10^3$ = trace. The amounts of each alkaloid are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two intensities are tabulated, two isomers are noted, and the intensities are in the order of elution from the GC column. Blanks indicate the alkaloid is not detected.
[c] Alkaloids reported for the first time. See Supplementary Information for characterization.

(Clark et al. 2005; Saporito et al., unpublished data). One *M. madagascariensis* from Ranomafana had two additional millipede alkaloids, the spiropyrrolizidines **222** and **252B**.

Individual Skin Alkaloid Analyses—M. madagascariensis from Besariaka Alkaloid composition of five extracts of *M. madagascariensis* from Besariaka (Table 4) was markedly different than those from *M. baroni* (Table 2) of the same site and from the *M. madagascariensis* from Ranomafana (Table 3). Alkaloid composition in *M. madagascariensis* of Besariaka was dominated by a previously unreported 5,6,8-trisubstituted-indolizidine **239Z** presumed to be of mite origin. A GC-FTIR spectrum was obtained. The Bohlmann band pattern indicated an indolizidine of the 5*Z*,9*Z* configuration with a non-hydrogen-bonded hydroxyl group (3,666 cm^{-1}). A tentative structure is presented in the Supplementary Information. Two minor isomers also were detected. Other putative mite alkaloids that were present in three of the five *M. madagascariensis* from Besariaka were the 1,4-disubstituted quinolizidine **217A** and the 5,6,8-trisubstituted indolizidines **223A** and **239C**. The 5,6,8-trisubstituted indolizidine **239Z** was not detected in *M. baroni* of Besariaka; however, both **217A** and **223A** were present. Accompanying the "izidine" alkaloids in *M. madagascariensis* of Besariaka were alloPTXs **305A**, **321C**, **323B**, and **325A**. On the basis of differences in the pattern of mass spectral fragmentation, it appears that some of the alloPTXs may prove to be 16-hydroxyl isomers of the usual 15-hydroxyl alloPTXs. Methoxy alloPTX alkaloids of molecular weight 337 were detected in three extracts. However, upon further analysis, these compounds appear to be artifacts of a chemical reaction of certain alloPTXs (probably **323B**) with methanol during exposure to HCl-methanol in the fractionation process. These apparent methoxy alkaloids were not detected when combined skin extracts of the five *M. madagascariensis* were partitioned using the standard method of Daly et al. (1994a, b) to yield an alkaloid fraction. Two indolizidines, namely the 3-butyl-5-methylindolizidine **195B** and the 3-hydroxybutyl analog **211E**, were detected in *M. madagascariensis* from Besariaka. These alkaloids are likely derived from myrmicine ants. Neither of these indolizidines was detected in the *M. baroni* from Besariaka.

Table 5 Alkaloid profiles for *Mantella bernhardi* (26 individuals) from Manombo, 1 February 2004

ZCMV# + sex[a]	SVL (mm)	Amount[b]	Pumiliotoxins				Izidines				
			Mites								
			251D (PTX)	**307F′** (PTX)	**309A** (PTX)	**325A** (aPTX)	**203A** (5,8-I)	**207I** (1,4-Q)	**217A** (1,4-Q)	**217B** (5,8-I)	**223X** (5,6,8-I)
502f	18.4	++							3	1	
503f	18.0	+++					1		3/1		
504f	17.8	++	1						1		
505m	15.6	++	3						2	1	
506f	18.1	+							3		
507m	16.0	++	3						2		
501m	14.9	+	3						2		
508m	16.5	++							3	2	2
509m	16.1	++	1						3		
510m	15.7	++	2	1	1				2		
520f	18.3	+++	1								
521f	19.2	+	3						3		
522f	18.1	++							2	2	
523f	17.3	+	1	1	2				2		
524f	18.0	++			3	2					
620f	18.3	++				1					3
526m	14.3	+	2						3/2		
527f	19.6	++	2					1	3/2		
528f	18.8	+++	2						3/2		
529m	16.6	+							3/2		
621f	17.8	++		1		1			1/2	1	
525m	15.1	+							2/1		
530m	16.3	++							3		
531f	17.2	++							2		
532m	15.6	++							3		
413s[d]	?	+++	3						1/1		

ZCMV# + sex[a]	Izidines								Spiros/tricyclics/unclass
	Mites				Ants				Millipedes/beetles/unknown
	231A (1,4-Q)	**233A** (1,4-Q)	**245F** (dehydro5,8-I)	**247O**[c] (dehydro5,8-I)	**249A** (3,5-I)	**251O** (3,5-P)	**267H** (3,5-P)	**275C** (3,5-I)	Other
502f			3		2				
503f			3	2	1				Tri **217H**[c](1); **231N** (1)
504f			1			3	2		Unclass **323H** (2)
505m			3			3			Tri **265CC**[c] (3)
506f			3		2	2			
507m	2		2			1			Unclass **237V**; **323H** (2)
501m	2		2			1			
508m	2	1	3		2	3			
509m	2		2	1		1	2		
510m	2		3		2	2			Unclass **235Q**(1); **237V** (1); **323H** (1)
520f					3			3	
521f			3		2	3			
522f					3	3		3	Unclass **323H** (3)
523f			2		2	1		1	Unclass **297F**[c] (3); **323H** (2)
524f			2		2	2		2	
620f					2	3	3	2	Unclass **323H** (2)
526m		1	1	1		2			

Table 5 (continued)

ZCMV# + sex[a]	Izidines								Spiros/tricyclics/unclass
	Mites				Ants				Millipedes/beetles/unknown
	231A (1,4-Q)	**233A** (1,4-Q)	**245F** (dehydro5,8-I)	**247O**[c] (dehydro5,8-I)	**249A** (3,5-I)	**251O** (3,5-P)	**267H** (3,5-P)	**275C** (3,5-I)	Other
527f			3		2	3			Unclass **151C**[c] (1); **235Q**(1)
528f		1	3	1		2			
529m			3		2	1			
621f						1			
525m			2		2	1			
530m			3		3	1		3	
531f	1		3		3	1		3	
532m	2		3	1		2/1			Unclass **231J** (1)
413s[d]			1			1			Unclass **235Q** (1); **323H** (1)

The probable class and dietary source of the alkaloid are indicated in the headings. The class of each alkaloid is indicated (see abbreviations).
[a] Sex (*m* male; *f* female) is indicated (*s* subadult).
[b] Alkaloid amounts [major (+++), minor (++), trace (+)] are based upon total ion chromatogram intensities with 10^4 or greater = major, 10^3–10^4 = minor; $\leq 10^3$ = trace. The amounts of each alkaloid are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two intensities are tabulated, two isomers are noted and the intensities are in the order of elution from the GC column.
[c] Alkaloids reported for the first time. See Supplementary Information for characterization.
[d] *UABD* uncataloged

Alkaloid Variation within and among Populations of M. madagascariensis With the exception of the one individual that contained no detectable alkaloids, alkaloid compositions among the six *M. madagascariensis* from Ranomafana were quite similar (see nMDS plot of Fig. 3b). The alkaloid compositions among the five individuals of *M. madagascariensis* from Besariaka were also similar to each other (see nMDS plot of Fig. 3b). Indeed, the alkaloid composition for two of the individual frogs was identical. However, alkaloid composition of the *M. madagascariensis* was significantly different between Ranomafana and Besariaka (Global R=1.0; P<0.008; Fig. 3b).

Individual Skin Alkaloid Analyses—M. bernhardi from Manombo The skin extracts of 26 *M. bernhardi* frogs collected from Manombo (Table 5) contained remarkably few alkaloids of the PTX group, with PTX **251D** as a dominant alkaloid in only five extracts and with PTX **309A** as a dominant alkaloid in only one extract. The alloPTX **325A** occurred as a minor alkaloid in one extract. The presumed mite alkaloids 1,4-disubstituted quinolizidines **217A** and **231A** and dehydro-5,8-disubstituted indolizidine **245F** were dominant alkaloids in most extracts. In addition, the putative ant 3,5-disubstituted pyrrolizidine **251O** and the 3,5-disubstituted indolizidines **249A** and **275C** occurred frequently as dominant alkaloids in many of the extracts. Interestingly, only one isomer of **275C** (5*Z*,9*Z* relative configuration) was observed in the extracts. This is in contrast to *M. baroni* (see Table 1), where frequently two or three diastereomers of **275C** were detected, with the 5,9*Z*-isomer usually being minor or absent. A minor diastereomer, accompanying 1,4-disubstituted quinolizidine **217A**, was seen in several extracts of the *M. bernhardi* from Manombo. This isomer of **217A** also occurred rarely in extracts of *M. baroni* (Tables 1 and 2). An isomer of alkaloid **217A** that previously had been detected in an extract of *Mantella betsileo* and based on comparison with synthetic material was shown to be the C-1-epimer of **217A** (unpublished results, cited in Michel et al. 2000).

The present collection of *M. bernhardi* from Manombo consisted of 11 males, 14 females, and 1 juvenile. This represents the only site in which numbers were large enough to compare alkaloid composition between sexes (see below). The 1,4-disubstituted quinolizidine **231A** appeared in six males, but in only one female. PTX **251D** was seen in 7 of the 11 males and only 4 of the 14 females. In the females, **251D** occurred as a trace alkaloid in three of these four individuals and as a minor alkaloid in only one individual. The 3,5-disubstituted indolizidine **275C** was observed in six females and in only one male. Thus, there appears to be a clear relationship between the occurrence of the putative mite alkaloid **231A** and of the putative ant alkaloid **275C** and the sex of the frog.

Individual Skin Alkaloid Analyses—M. bernhardi from Vevembe In contrast to the izidine-dominated alkaloid compositions for the 26 *M. bernhardi* from Manombo (Table 5), the compositions for the 25 *M. bernhardi* of Vevembe were dominated by alkaloids of the PTX group (Table 6). Nearly all individuals had PTXs **251D** and **309A**

Table 6 Alkaloid profiles for *Mantella bernhardi* (25 individuals) from Vevembe, 10 February 2004

ZCMV# + sex[a]	SVL (mm)	Amount[b]	Pumiliotoxins/homopumiliotoxins							
			Mites							
			251D (PTX)	**253A** (aPTX)	**253F** (PTX)	**267C** (PTX)	**293D** (deoxyPTX)	**307F′** (PTX)	**307F″/F‴** (PTX)	**307K**[c] (deoxyhPTX)
701m	15.8	+++						1		
702m	15.4	+++	3							
703m	15.1	+++	3							
704m	15.4	+++	3			1				
705m	15.5	+++	3					1	1/1	
706m	14.4	+++	1					2	1/1	
707m	14.2	+++	3							
708m	15.4	+++	3							
709m	15.7	+++	3							
710f	17.4	+	3							
711f	17.2	+++	3							
712m	16.0	+++	3							
713m	14.3	+++	3					2		
714m	15.5	++						2	1/1	
715m	14.0	+++	3			1				
901m	15.2	+++	3			1		1	1/1	
902m	14.6	+++	2					1	1/1	
903f?	18.2	+++	3							
904f?	18.6	+++	3					1		
905m	15.4	+++	3			1				
906m	15.4	+++						1		1
907m?	16.1	+++	3			1				
908m	16.3	+++	3				1	1		1
909m	14.8	+++	3	1	1	1				
m	15.9	+++	3			1				

ZCMV# + sex[a]	Pumiliotoxins/homopumiliotoxins						Izidines			
	Mites									
	309A (PTX)	**321B** (hPTX)	**323A** (PTX)	**323E** (hPTX)	**323J**[c] (aPTX)	**325A** (aPTX)	**217A** (1,4-Q)	**223X** (5,6,8-I)	**231A** (1,4-Q)	**235B″** (5,8-I)
701m	3			3		3				
702m			1			3				
703m						2				
704m					1	2				
705m	2	1/1		1	1	3		1		
706m	3	1		2	1	2		2		
707m	1					2				
708m					1	2				
709m		1			1	3			2	
710f					1	3				
711f					1	2		1		
712m					1	2				
713m	2			2	1	2				
714m	2	1		2		2				
715m	1					2		2		
901m					1	2				1
902m	3			2		1	1			2
903f?					1	1/2				
904f?	2			1	1	2				

Table 6 (continued)

ZCMV# + sex[a]	Pumiliotoxins/homopumiliotoxins						Izidines			
	Mites									
	309A (PTX)	**321B** (hPTX)	**323A** (PTX)	**323E** (hPTX)	**323J**[c] (aPTX)	**325A** (aPTX)	**217A** (1,4-Q)	**223X** (5,6,8-I)	**231A** (1,4-Q)	**235B″** (5,8-I)
905m						2				
906m	3			3		3				
907m?	1			2	1	2				
908m	3			2		2		3		
909m	1					2		1		
m	1					2				

ZCMV# + sex[a]	Izidines							Spiros/tricycles/unknown
	Mites		Ants					Millipedes/beetles/ unclass
	245F (dehydro5,8-I)	**247O**[c] (dehydro5,8-I)	**237R** (3,5-P)	**239BB**[c] (3,5-P)	**249A** (3,5-I)	**251K** (3,5-P)	**251O** (3,5-P)	Other
701m					2			
702m	2	2					2	
703m								
704m			1				1	
705m			1	1			1	
706m							1	
707m								
708m								
709m	2							
710f							2	
711f							1	
712m								
713m			1					
714m								
715m						1		
901m								
902m								
903f?				2/1			1/1	Unclass **231J** (1)
904f?				1			2/2	
905m							2	
906m				1				
907m?				1				
908m				1			2/1	
909m				1/1			1	
m				1/1			1	Unclass **231J** (1)

The probable class and dietary source of alkaloids are indicated (see abbreviations).

[a] Sex: *m* male; *f* female; *?* indicates uncertainty as to sex.

[b] Total content of alkaloids [major (+++), minor (++), trace (+)] is based upon total ion chromatogram intensities with 10^4 or greater = major, 10^3–10^4 = minor, $\leq 10^3$ = trace. The amounts of each alkaloid in the table are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two intensities are tabulated, two isomers are noted, and the intensities are in the order of elution from the GC column. Blanks indicated that the alkaloid was not detected.

[c] Alkaloids reported for the first time. See Supplementary Information for characterization.

and alloPTX **325A** as significant or dominant alkaloids. However, three frogs had no PTX **251D**. HomoPTX **323E** was present in eight frogs as a significant alkaloid. A previously unreported alloPTX **323J** was present in trace amounts in 13 frogs. Another previously unreported alkaloid, deoxy-homoPTX **307K**, was found in two frogs. In contrast to the *M. bernhardi* from Manombo, "izidine" alkaloids that are proposed to be derived from dietary mites occurred rarely in *M. bernhardi* from Vevembe. Thus, the 1,4-disubstituted quinolizidine **217A** and the dehydro-5,8-

Table 7 Summary of alkaloids detected in single-skin extracts of populations of *Mantella*

Table number		1	2	3	4	5	6
Mantella species		*M. baroni*		*M. madagascariensis*		*M. bernhardi*	
Alkaloids		Ran.	Bes.	Ran.	Bes.	Man.	Vev.
PTX	**237A**	x			x		
	251D	x	x	x	x	x	x
	253F						x
	267C	x	x		x		x
	307A		x				x
	307F	x	x/x/x			x	x/x/x
	307G		x				
	309A	x	x/x	x	x	x	x
	323A				x		x
aPTX	**253A**			x			
	305A			x			
	305C			x			
	321C			x			
	323B		x	x/x			x
	323J[a]		x				x
	325A	x	x/x			x	x/x
deoxy PTX	**251H**	x			x		
	265X		x				
	281N		x				
	293D	x					x
hPTX	**251R**	x			x		
	265N	x/x			x		
	281K	x			x/x		
	321B						x/x
	323E						x
d-hPTX	**307K**[a]						x
3,5-P	**197J**[a]				x		
	223M		x				
	237R						x
	239K				x		x
	239BB[a]						x/x
	249I	x					
	251K						x
	251O	x/x	x		x	x/x	x/x
	263S[a]				x		
	265W	x			x		
	267H	x			x	x	
3,5-I	**195B**			x			
	211E			x			
	249A	x			x	x	x
	275C	x/x/x			x/x	x	
5,8-I	**203A**				x	x	
	217B	x			x	x	
	235B″						x
	247E		x				
	251N	x	x		x		
	267E		x	x			
	267S		x	x	x		
dehydro 5,8-I	**205L**[a]	x					
	207W[a]				x		
	245F		x			x	x
	247O[a]					x	x
	265F		x				

Table 7 (continued)

Table number		1	2	3	4	5	6
Mantella species		*M. baroni*		*M. madagascariensis*		*M. bernhardi*	
Alkaloids		Ran.	Bes.	Ran.	Bes.	Man.	Vev.
	293O[a]	x			x		
5,6,8-I	**223A**		x	x			
	223X					x	x
	239C			x			
	239Z[a]			x/x			
	249C		x				
	249BB[a]		x				
	265L		x				
	265U		x				
	269J[a]			x			
	273A	x/x			x/x		
	289G[a]				x		
1,4-Q	**207I**					x	
	217A	x/x	x/x	x	x	x/x	x
	231A	x/x	x		x	x	x
	233A	x	x			x	
Spiro	**222**				x		
	236	x			x/x		
	252B				x		
Tricyclics	**217H**[a]					x	
	231N[a]					x	
	245J		x				
	247N[a]				x		
	263M				x		
	263T[a]				x		
	265CC[a]					x	
Izidines	**211B**			x			
	291J				x		
	309K[a]				x		
Unclass.	**151C**[a]					x	
	183C				x		
	197D				x		
	199B[a]				x		
	207N	x					
	231J					x	x
	235Q					x	
	237V[a]					x	
	249AA[a]		x				
	297F[a]					x	
	307L[a]		x		x		
	323H					x	

For detailed distribution and quantitation data, see Tables 1, 2, 3, 4, 5, and 6. Structures for selected alkaloids are shown in Fig. 2 (see also Daly et al. 2005). See abbreviations for alkaloid classes.
Ran. Ranomafama; *Bes.* Besariaka; *Man.* Manombo; *Vev.* Vevembe; *d-hPTX* deoxy-hPTX
[a] Previously undescribed alkaloids. Properties and tentative structures for some of these alkaloids are in the Supplementary Information.

disubstituted indolizidine **245F** were common and often dominant alkaloids in *M. bernhardi* from Manombo (Table 5), but these alkaloids occurred rarely in *M. bernhardi* from Vevembe, with **217A** being found as a trace alkaloid in one frog and **245F** being found in significant amounts in only two frogs (Table 6). "Izidine" alkaloids that are presumably derived from dietary ants were represented in the Vevembe frogs by four 3,5-disubstituted pyrrolizidines, namely **237R**, **239BB**, **251K**, and **251O** and by the 3,5-disubstituted indolizidine **249A** (diastereomers of these alkaloids were occasionally present). Of these 3,5-disubstituted pyrrolizidines, only **239BB**

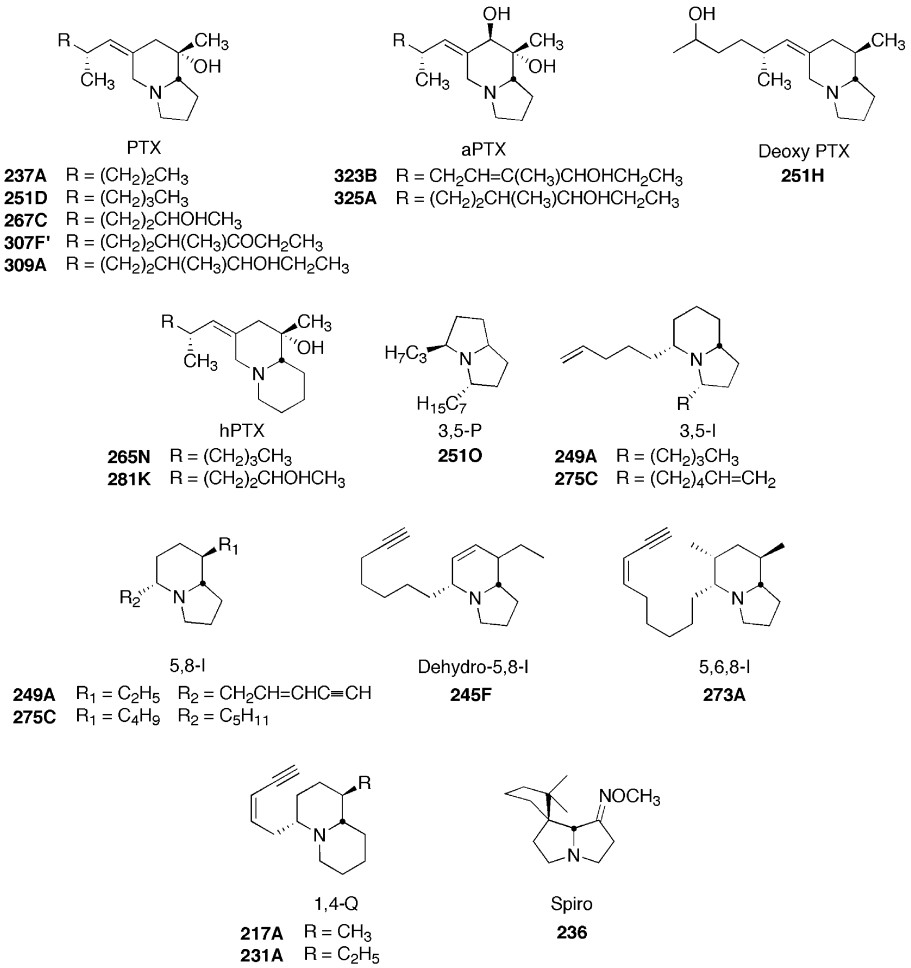

Fig. 2 Representative alkaloids of 11 of the structural classes detected in the present collections of mantellid species. These alkaloids occur relatively frequently or are major alkaloids in several extracts as are the following new alkaloids: deoxy-homoPTX **307K***, 3,5-disubstituted pyrrolizidine **239BB***, and 5,6,8-trisubstituted indolizidine **239Z*** (for tentative structures, see Supplementary Information)

and **251O** occurred in several frogs, and overall, such putative ant alkaloids were not very common in *M. bernhardi* from Vevembe. In the *M. bernhardi* from Manombo, the putative ant alkaloids **249A** and **251O** were present in almost all frogs (Table 5).

Alkaloid Variation Within and Among Populations of M. bernhardi There was a somewhat uniform variation in alkaloid composition among the 26 individuals of *M. bernhardi* from Manombo and also among the 25 individuals of *M. bernhardi* from Vevembe (see nMDS plot of Fig. 4a). There did appear to be a group of eight frogs from Vevembe (nos. 903 f to 910 m of Table 6) that were characterized by an alkaloid mix of PTX **309A** and izidines **239BB** and **251O** along with the very common PTX **251D** and alloPTX **325A**. The alkaloid composition of *M. bernhardi* was significantly different between Manombo and Vevembe (Global R=0.83; P<0.001; Fig. 4a). The alkaloid composition of *M. bernhardi* from Manombo was not significantly different between sexes (Global R=0.06; P= 0.20; Fig. 4b).

Alkaloid Variation among Species Alkaloid composition was significantly different between *M. baroni* and *M. madagascariensis* from Besariaka (Global R=1.0; P< 0.008; Fig. 5a). Alkaloid composition was also significantly different between *M. baroni* and *M. madagascariensis* from Ranomafana (Global R=0.96; P<0.001; Fig. 5b). A summary of variation is provided in Table 7.

Combined Skin Alkaloid Analyses—M. baroni A variety of PTXs (Table 8) and izidine alkaloids (Table 9) were detected among the five populations of *M. baroni*. The six combined skins from Mangevo (Fig. 1) and the one skin from Vohiparara (Kidonavo Bridge location of Fig. 1) are from the Ranomafana National Park. Alkaloid composition for the combined six skins of *M. baroni* from Mangevo was similar to those of the individual *M. baroni* frogs of

Table 8 Alkaloid profiles of pumiliotoxins/homopumiliotoxins for five populations of *M. baroni* from Mangevo (six skins), Andriabe (two skins), Tsinjoarivo (two skins), Vohidrazana (two skins), and Vohiparara (one skin), Jan–Feb 2003 (see Table 9 below for izidines and other alkaloids)

Species/site	Pumiliotoxins/homopumiliotoxins						
	Mites						
	Amount[a]	**237A** (PTX)	**251D** (PTX)	**251H** (deoxyPTX)	**265N** (hPTX)	**267C** (PTX)	**267N** (deoxyhPTX)
M.baroni/Mangevo #1	+++	3	2				
M. baroni/Andriabe	+++		2				1
M. baroni/Tsinjoarivo	+++						
M. baroni/Vohindrazana	+++	1	1				
M. baroni/Vohiparara	+++	1	3	1	2	1	

Species/site	Pumiliotoxins/homopumiliotoxins						
	Mites						
	279L[b] (desMePTX)	**281F** (H_2-PTX)	**291E** (deoxyPTX)	**293D** (deoxyPTX)	**295C** (deoxyPTX)	**307F′** (PTX)	**307F″/307F‴** (PTX)
M.baroni/Mangevo #1				1		2	
M. baroni/Andriabe						1	
M. baroni/Tsinjoarivo	1		2	1/1	2	1	1/1
M. baroni/Vohindrazana		2	2	1/2		1	1/1
M. baroni/Vohiparara						1	

Species/site	Pumiliotoxins/homopumiliotoxins					
	Mites					
	307G (PTX)	**309A** (PTX)	**323J** [b] (aPTX)	**323E** (hPTX)	**325A** (aPTX)	**337A** (hPTX)
M.baroni/Mangevo #1	2	3	1		2	1
M. baroni/Andriabe	1	3	2		3	
M. baroni/Tsinjoarivo	2	3		1	2	
M. baroni/Vohindrazana	3	3/1		1	1	
M. baroni/Vohiparara		2			1	

See Fig. 1 for sites and Supplementary Information for GPS coordinates, elevations, and exact dates of collection. The probable classes and dietary source are indicated in the heading.

[a] Total content of alkaloids is major (+++) in each case (see definition in legend to Table 6). The amounts of each alkaloid in the table are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two intensities are tabulated, two isomers are noted, and the intensities are in the order of elution from the GC column. Blanks indicate the alkaloid is not detected.

[b] Alkaloids reported for the first time. See Supplementary Information for characterization.

Ranomafana (Ranomafanakely location of Fig. 1). The presence of PTXs **237A**, **251D**, **307F′**, and **309A** and alloPTX **325A** in the combined skins from Mangevo (Table 8) and individual skins (Table 1) reflect similarities in alkaloid composition for these two populations of *M. baroni*. The absence of homoPTX **265N** and the presence of PTX **307G** in substantial amounts only in the combined skins represent differences for these two populations of *M. baroni*. The differences in alkaloids among the populations of combined skins from Mangevo and individual skins of *M. baroni* from Ranomafana, particularly the lack of detection of **307G** and **223B** in the 15 individual skins, likely reflect differences in the geographic location of the two frog populations (Fig. 1). The alkaloids from five populations of *M. baroni* in Tables 8 and 9 that seem most common were the putative mite alkaloids PTXs **251D**, **307F′**, **307G**, and **309A**, alloPTX **325A**, and 1,4-disubstituted quinolizidines **217A** and **231A**, whereas the most

Table 9 Profiles of "izidines" and other alkaloids for five populations of *M. baroni* from Mangevo (six skins), Andriabe (two skins), Tsinjoarivo (two skins), Vohindrazana (two skins), and Vohiparara (one skin), Jan–Feb 2003

Species/site	Izidines								
	Mites								
	217A (1,4-Q)	**217B** (5,8-I)	**231A** (1,4-Q)	**231K** (5,6,8-I)	**233A** (1,4-Q)	**235B″** (5,8-I)	**241F** (5,8-I)	**243C** (5,8-I)	**245B** (5,8-I)
M. baroni/Mangevo #1	2		2						
M. baroni/Andriabe	2	2	2	1	2	2	1/2	2	2/2
M. baroni/Tsingoarivo	3								
M. baroni/Vohindrazana	2		2				1	2	
M. baroni/Vohiparara	1	1	1/2						

Species/site	Izidines								
	Mites								
	245H (dehydro-5,8-I-I)	**247E** (5,8-I)	**247F** (5,8-I)	**247J** (izidine)	**251N** (5,8-I)	**251V** (5,6,8-I)	**253B** (5,8-I)	**257D** (1,4-Q)	**273A** (5,6,8-I)
M. baroni/Mangevo #1	1			1					
M. baroni/Andriabe							1		
M. baroni/Tsingoarivo		2						1	
M. baroni/Vohindrazana			1			1		1	1
M. baroni/Vohiparara					1				1

Species/site	Izidines						Spiros/tricyclics/unclass
	Mites		Ants				Millipedes/beetles/unknown
	279D (5,8-I)	**281I** (5,8-I)	**223B** (3,5-P)	**249A** (3,5 I)	**251O** (3,5-P)	**275C** (3,5-I)	Other Alkaloids
M. baroni/Mangevo #1			2	1	2	1	
M. baroni/Andriabe					2	2/2	Tri **245J** (2); unclass **307M**[a] (*1*)
M. baroni/Tsingoarivo	1			2		3/2	Tri **243G** (2), **245J** (*2*); unclass **357B** (1)
M. baroni/Vohindrazana	3/1	1		3	1	2/1	Unclass **323G** (2)
M. baroni/Vohiparara						1/1	Unclass **231J** (1), **235R** (1), **249P** (1)

See Fig. 1 for sites and Supplementary Information for GPS coordinates, elevations, and exact dates of collection. The probable classes and dietary source are indicated in the heading.

Total content of alkaloids is major (+++) in each case (see definition in legend to Table 6). The amounts of each alkaloid in the table are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two intensities are tabulated, two isomers are noted, and the intensities are in the order of elution from the GC column. Blanks indicate the alkaloid is not detected.

[a] Alkaloids reported for the first time. See Supplementary Information for characterization.

common putative ant alkaloids were the pyrrolizidine **251O** and indolizidine **275C**.

Combined Skin Alkaloid Analyses—M. bernhardi PTXs **237A** and **309A**, homoPTX **337A**, 1,4-disubstituted quinolizidines **217A** and **231A**, 5,8-disubstituted indolizidine **245A**, dehydro-5,8-I **245H**, "izidine" **247J**, and 3,5-disubstituted pyrrolizidines **223B** and **251O** occurred both in *M. bernhardi* from Mangevo and in a nearby population of *M. baroni* also from Mangevo (Table 10, data on *M. baroni* is repeated from Tables 8 and 9). The *M. bernhardi* also contained a remarkable number of 5,8-disubstituted indolizidines, 1,4-disubstituted quinolizidines, and unclassified alkaloids, of which only a few were detected in the nearby population of *M. baroni*.

Combined Skin Alkaloid Analyses—M. cowanii Two skins of *M. cowanii*, a species closely related to *M. baroni* (Chiari

Table 10 Alkaloid profiles for *Mantella baroni* (six skins) and *M. bernhardi* (eight skins) both from the Mangevo area and *M. cowani* (two skins) from Antoetra, Jan–Feb 2003

Species/site	Amount[a]	Pumiliotoxins/homopumiliotoxins										
		Mites										
		237A (PTX)	**251D** (PTX)	**251H** (deoxyPTX)	**265G** (PTX)	**267C** (PTX)	**277B** (PTX)	**291G** (PTX)	**293D** (deoxyPTX)	**307F′** (PTX)	**307G** (PTX)	**309A** (PTX)
M. baroni/ Mangevo #1	+++	3	2						1	2	2	3
M. bernhardi/ Mangevo #2	+++	1										2
M. cowanii/ Antoetra	+++		3	1		1	1	1		1	1	2

Species/site	Pumiliotoxins/homopumiliotoxins					Izidines						
	Mites											
	323A (PTX)	**323J**[b] (aPTX)	**325A** (aPTX)	**337A** (hPTX)	339D (aPTX)	**205A** (5,8-i)	**217A** (1,4-Q)	**217B** (5,8-I)	**219B** (1,4-Q)	**231A** (1,4-Q	**221I** (5,8-I)	**241F** (5,8-I)
M. baroni/ Mangevo #1		1	2	1			2			2		
M. bernhardi/ Mangevo #2				1	2/2	1	3/2		1	1	1	1/1
M. cowanii/ Antoetra	1	1	2					2				

Species/site	Izidines										Tricycles/unclass
	Mites						Ants				Beetles/unknown
	243B (5,8-I)	**245A** (5,8-I)	**245H** (dehydro-5,8-I)	**247J** (izidine)	**249O** (5,8-I)	**279F** (5,6,8-I)	**223B** (3,5-P)	**249A** (3,5-I)	**251O** (3,5-P)	**275C** (3,5-I)	Other
M. baroni/ Mangevo #1		1	1	1			2	1	2	1	
M. bernhardi/ Mangevo #2	1	1	1	1	1	1	3		2		Tri. **235M** (1), unclass: **275J** (2/2), **293J** (2), **323H** (2), **325C** (1), **339E** (3/3),**341D** (1), **369** (2), **371** (2/2)
M. cowanii/ Antoetra		1									Unclass. **231J** (1) **249P** (1), **390** (1)

The probable class and dietary source of each alkaloid are indicated in the headings (see abbreviations).
[a] Total content of alkaloids is major (+++) in each case, indicating a total ion chromatogram intensity of 10^4 or greater. The amounts of each alkaloid in the table are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Blanks indicate the alkaloid is not detected. Where two intensities are tabulated, two isomers are noted and the intensities are in the order of elution from the GC column.
[b] Alkaloids reported for the first time. See Supplementary Information for characterization.

et al. 2005), contained a variety of PTXs (Table 10). The dominant alkaloid was PTX **251D**. An earlier study of *M. cowanii*, frogs of which were presumably collected near Antoetra, reported PTX **251D** as the predominant alkaloid (Daly et al. 1996). The current *M. cowanii* had remarkably few izidines, consisting only of significant amounts of the 5,8-disubstituted indolizidine **217B** and trace amounts of the 5,8-disubstituted indolizidine **245A**.

Number of Alkaloids vs. Frog Size for Three Mantellid Species There was a positive significant relationship between the number of alkaloids and frog size (measured as

Table 11 Summary of alkaloids detected in extracts of populations of *Mantella*

Table number		8 and 9					10	10
Mantella species		*M. baroni*					*M. bernhardi*	*M. cowanii*
Alkaloids		Man. 1	And.	Tsi.	Vdr.	Vpa.	Man. 2	Ant.
PTX	**237A**	x			x	x	x	
	251D	x	x		x	x		x
	265G							x
	267C					x		x
	277B							x
	291G							x
	307F	x	x	x/x/x	x/x/x	x		x
	307G	x	x	x	x			x
	309A	x	x	x	x/x	x	x	x
	323A							x
aPTX	**323J**[a]			x				x
	325A	x	x	x	x	x		x
	339D						x/x	
deoxy PTX	**251H**					x		x
	291E			x	x			
	293D			x/x	x/x			
	295C			x				
dm-PTX	**279L**[a]			x				
H2-PTX	**281F**				x			
hPTX	**265N**					x		
	323E			x	x			
	337A	x					x	
dm-hPTX	**267N**		x					
3,5-P	**223B**	x					x	
	251O	x	x		x		x	
3,5-I	**249A**	x		x	x			
	275C	x	x/x	x/x	x/x	x/x		
5,8-I	**205A**						x	
	217B		x			x		x
	221I						x	
	235B″		x					
	241F		x/x				x/x	
	243B						x	
	243C		x					
	245B		x/x				x	
	247E			x				
	247F				x			
	249O						x	
	251N					x		
	253B		x					
	279D			x	x/x			
	281I					x		
dh-5,8-I	**245H**	x					x	
5,6,8-I	**231K**		x					
	251V				x			
	273A				x	x		
	279F						x	
1,4-Q	**217A**	x	x	x	x	x	x/x	
	219B						x	
	231A	x	x		x	x/x	x	
	233A		x					
	257D			x	x			
Tricyclics	**235M**						x	

Table 11 (continued)

Table number		8 and 9					10	10
Mantella species		*M. baroni*					*M. bernhardi*	*M. cowanii*
Alkaloids		Man. 1	And.	Tsi.	Vdr.	Vpa.	Man. 2	Ant.
	243G			x				
	245J		x	x				
Izidines	**247J**						x	
Unclass.	**231J**					x		x
	235R					x		
	249P					x		x
	275J						x	
	293J						x	
	307M[a]		x					
	323G				x			
	323H						x	
	325C						x	
	339E						x	
	341D						x	
	357B			x				
	369						x	
	371						x	
	390							x

Structures for selected alkaloids are shown in Fig. 2 (see also Daly et al. 2005). For detailed distribution and quantitation data, see Tables 8, 9 and 10. See abbreviations for alkaloid classes.

[a] Previously undescribed alkaloids. Properties and tentative structures for some of these alkaloids are in the Supplementary Information.

Man. 1 Mangevo 1; *Man. 2* Mangevo 2; *And.* Andriabe; *Tsi.* Tsinjoarivo; *Vdr.* Vohindrazana; *Vpa.* Vohiparara; *Ant.* Antoetra, *dm-PTX* desmethyl-PTX, *H2-PTX* dihydro-PTX, *dm-hPTX* desmethyl-hPTX, *dh-5,8-l* dehydro-5,8-l.

SVL), when all three species of mantellids were examined together (Fig. 6; $P<0.001$; $R^2=0.37$). However, when each species was examined independently, only *M. baroni* had a positive significant relationship between the number of alkaloids and frog size (Fig. 6; $P<0.02$; $R^2=0.27$). There was no relationship between the number of alkaloids and frog size for *M. madagascariensis* and *M. bernhardi* (Fig. 6; $P=0.838$ and $P=0.975$, respectively).

Discussion

Alkaloid composition varied within and among species of the mantellid frogs examined in this study (see MDS plots of Figs. 3, 4 and 5). Differences in alkaloid composition within species were largely related to geographic location, with the same species differing significantly in alkaloid composition between locations. These findings suggest that there are differences in the availability of alkaloid-containing arthropods based on geographic location, which are likely influenced by differences in habitat (i.e., vegetation, leaf litter, forest structure, etc.) at each location. Differences in alkaloid composition among locations have been reported previously for various poison frogs, including frogs of the genera *Mantella* (Garraffo et al. 1993a; Daly et al. 1996; Clark et al. 2005, 2006), *Melanophryniscus* (Garraffo et al. 1993b; Mebs et al. 2005; Daly et al. 2007), and *Pseudophryne* (Daly et al. 1990; Smith et al. 2002), as well as the dendrobatid frogs (Daly et al. 1987; Myers et al. 1995; Saporito et al. 2006, 2007b; and numerous references within). Within the same location, marked individual differences in alkaloid composition of the same species were also observed in the present study. Although these differences were not as great as differences between locations, they certainly suggest that location and availability of alkaloid-containing arthropods are also important on small spatial scales. Insights into the factors that result in differences in alkaloid composition among individuals from the same geographic location remain a challenge for further research.

The early studies on the alkaloid composition for collections of a mantellid species [e.g., *M. aurantiaca*, *M baroni*, *M. crocea*, and *M. pulchra*] at the same location indicated that there can be marked differences in alkaloid composition over time (Garraffo et al. 1993a; Daly et al. 1996). Recently, further examples of apparent temporal variation for populations of *M. baroni* from Sahavondrana and Vatoharanana locations in the Ranomafana region have been reported (Clark et al. 2006). Such findings indicate

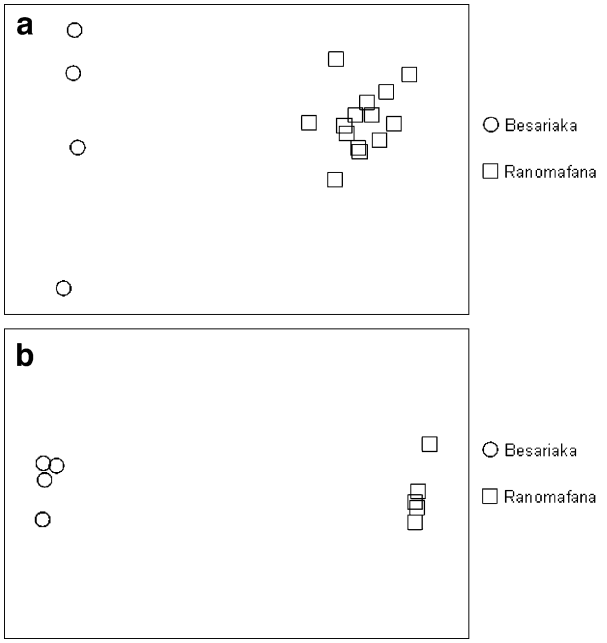

Fig. 3 **a** Multidimensional scaling plot of alkaloid composition in *M. baroni* from two different locations in Madagascar (stress=0.08). Alkaloid composition of *M. baroni* is significantly different between locations (Global R=0.99; P<0.001). *Each symbol* represents an individual frog from one of the two locations. The *distance between symbols* represents the difference in alkaloid composition. One subadult frog from Ranomafana (Table 1, no. 128s) was removed from the analysis due to the small number of alkaloids (only two in trace amounts) detected relative to other frogs. **b** Multidimensional scaling plot of alkaloid composition in *M. madagascariensis* from two different locations in Madagascar (stress=0.01). Alkaloid composition of *M. madagascariensis* is significantly different between locations (Global R=1.0; P<0.008). *Each symbol* represents an individual frog from one of the two locations. The *distance between symbols* represents the difference in alkaloid composition. One male frog from Ranomafana (Table 3, no. 112m) was removed from the analysis due to the absence of detectable alkaloids

that, in addition to geographic location, time also plays a prominent role in alkaloid variation of mantellid frogs. Temporal variation also has been reported for dendrobatid frogs (Daly et al. 1987, 2002; Saporito et al. 2006, 2007b) and bufonid toads (Daly et al. 2007). Such differences in alkaloid composition over time would likely be because of successional changes in habitat (i.e., vegetation, leaf-litter, forest structure, etc.), because of factors such as disturbance, which in turn lead to temporal shifts in alkaloid-containing arthropod availability (also see Daly et al. 2000; Saporito et al. 2006, 2007b).

Alkaloid profiles for three individuals of *M. baroni* collected in 2003 at Vatoharanana (see Fig. 1) were reported by Clark et al. (2005, 2006). Based on the GC–MS chromatograms in the Supplementary Information of Clark et al. (2006), the major alkaloids in all three frogs (in order of elution from the GC column) were as follows: quinolizidine **217A**, indolizidine **217B**, PTX **237A** and PTX **309A**. Other alkaloids, present as significant constituents in one or two individuals, were quinolizidines **233A** and **257D**, PTXs **251D** and **307G**, indolizidine **275C**, and pyrrolizidine **251O**. Except for the last two putative ant alkaloids, all the major alkaloids are of putative mite origin. It should be noted that, often, the alkaloid peaks in the GC–MS traces are listed incorrectly in Table 1 of Clark et al. (2006). For example, PTX **309A**, a major alkaloid in the GC–MS traces for all three individuals, is listed as minor or trace in the table. The alkaloid composition for a combined sample of ten skins of *M. baroni* collected in Ranomafana in November of 1989 probably at or near the same Vatoharanana site was reported to consist mainly of PTX **309A** and 1,4-disubstituted quinolizidine **217A**, with significant amounts of PTX **237A**, 1,4-disubstituted quinolizidines **231A** and **233A**, and 5,8-disubstituted indolizidines **217B**, **243D**, and **245C** (Daly et al. 1996; see also Clark et al. 2006). Thus, with respect to the major alkaloid components, there did not appear to be major temporal changes.

A significant variation in alkaloid composition was observed among sympatric species sampled at the same location (see nMDS plots of Fig. 5a, b and Tables 1, 2, 3, and 4). *M. madagascariensis* and *M. baroni* were collected together at two different locations (Ranomafana [Ranoma-

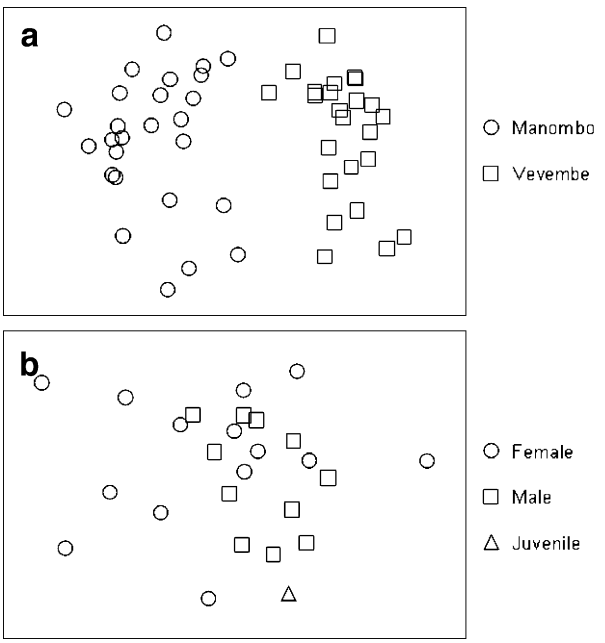

Fig. 4 **a** Multidimensional scaling plot of alkaloid composition in *M. bernhardi* from two different locations in Madagascar (stress=0.13). Alkaloid composition of *M. bernhardi* is significantly different between locations (Global R=0.83; P<0.001). *Each symbol* represents an individual frog from one of the two locations. The *distance between symbols* represents the difference in alkaloid composition. **b** Multidimensional scaling plot of alkaloid composition between sexes of *M. bernhardi* from Manombo (stress=0.17). Alkaloid composition is not significantly different between sexes (Global R=0.06; P=0.20). One of the frogs (Table 5, no. 413s) in the sample is a subadult. *Each symbol* represents an individual frog. The *distance between symbols* represents the difference in alkaloid composition

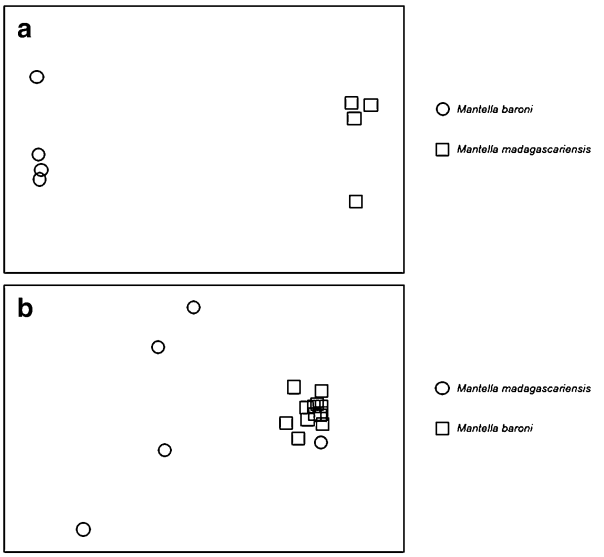

Fig. 5 **a** Multidimensional scaling plot of alkaloid composition in *Mantella baroni* and *M. madagascariensis* from Besariaka (stress= 0.01). Alkaloid composition is significantly different between *M. baroni* and *M. madagascariensis* (Global R=1.0; P<0.008). *Each symbol* represents an individual frog from one of the two species. The *distance between symbols* represents the difference in alkaloid composition. **b** Multidimensional scaling plot of alkaloid composition in *M. madagascariensis* and *M. baroni* from Ranomafana (stress= 0.1). Alkaloid composition is significantly different between *M. madagascariensis* and *M. baroni* (Global R=0.798, P<0.001). One male *M. madagascariensis* frog (Table 3, no. 112m) was removed from the analysis due to the absence of detectable alkaloids. One subadult *M. baroni* frog (Table 1, no. 128s) was removed from the analysis due to the small number of alkaloids (only two in trace amounts) detected relative to other frogs. *Each symbol* represents an individual frog from one of the two species. The *distance between symbols* represents the difference in alkaloid composition

fanakely] and Besariaka; see Fig. 1) and differed significantly between each other in alkaloid composition within each of the locations. As explained in "Materials and Methods," the two species may use partly different microhabitats at the Ranomafana site, but apparently not at the Besariaka site. Differences in alkaloid composition among these two sympatric species suggest that, at least in the case of Besariaka, there are either differences in feeding or in the sequestering systems responsible for uptake of alkaloids. Interestingly, these two sympatric species were originally considered different color morphs of the same species, but recent genetic analyses have suggested that they are separate, not even closely related species that belong to different species groups (Chiari et al. 2004, 2005). Certainly, the differences in alkaloid composition among these two sympatric species complement the findings that these are two different species. Future dietary analyses and alkaloid feeding experiments with these two species would provide more information as to whether or not alkaloid variation is because of differences in feeding or in the uptake system that sequesters dietary alkaloids. Whether there may also be changes in dietary habits with the age of the frogs is unknown.

Alkaloid composition for one individual of *M. madagascariensis*, which was collected in 2003 along with six *M. baroni* from Vohiparara (at Kidonavo bridge in Fig. 1), has been reported (Clark et al. 2005, 2006). Based on the GC–MS chromatograms in the Supplementary Information of Clark et al. (2006), the major alkaloids in the *M. madagascariensis* sample (in order of elution from the GC column) appear to be the following: indolizidines **203A**, **205A**, and **217B**; quinolizidine **217A**; an unidentified (not listed by Clark et al. 2006) alkaloid, which is likely the dehydro-5,8-disubstituted indolizidine **205L**, reported as a

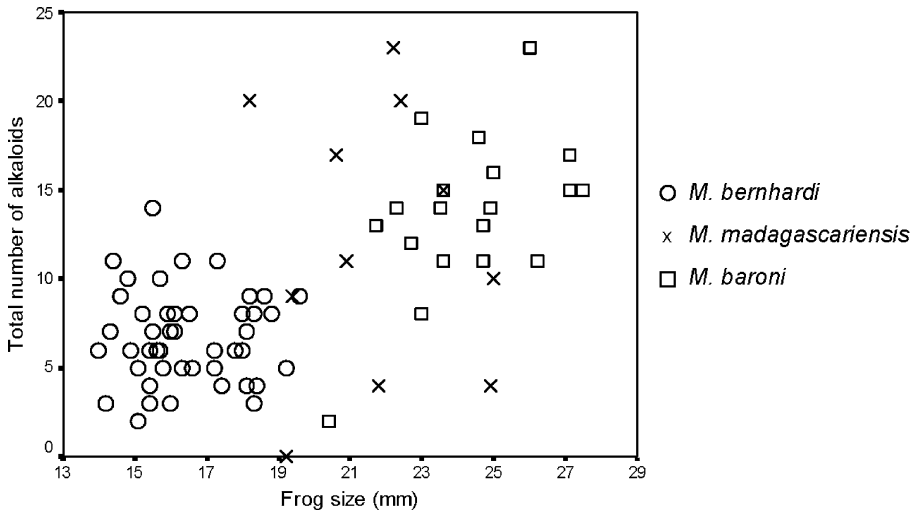

Fig. 6 The number of alkaloids versus frog size for all three mantellid species. When all three species are examined together, there is a positive significant relationship between number of alkaloids and frog size (P<0.001; R^2=0.37). However, when each species is examined independently, only *M. baroni* has a positive significant relationship between number of alkaloids and frog size (P value=0.02; R^2=0.27). There is no relationship between the number of alkaloids and frog size for *M. madagascariensis* and *M. bernhardi*

new alkaloid for the first time in the present study; homoPTX **265N**; indolizidine **275E**; and PTX **267C** (designated in error in the GC–MS legend of Clark et al. 2006 as deoxyPTX **267N**). All of these major alkaloids are probably of mite origin. The profiles of alkaloids in the six *M. baroni* from the same site were quite different from the one *M. madagascariensis* frog, based on the GC–MS chromatograms in the Supplementary Information of Clark et al. (2006). The dominant alkaloids were PTXs **251D**, **307G**, and **309A**, homoPTX **265N**, and for certain individuals pyrrolizidine **251O** and indolizidine **273A**. It should be noted that, often, minor or trace peaks in the GC–MS traces are listed in Table 1 of Clark et al. (2006) as major alkaloids (see critical comments on the Clark data in the Supplementary Information for the present report).

Differences in alkaloid composition between sexes have been suggested for other poison frogs (see Saporito et al. 2006, 2007b); however, because of small sample sizes and the confounding effects of location, this has not been specifically examined for any species. In the present study, an examination of alkaloid profiles in *M. bernhardi* from Manombo (Table 5: 11 males, 14 females) provided an opportunity to examine alkaloid composition between sexes. Alkaloid composition was not found to be significantly different between sexes (see nMDS plot in Fig. 4b; $P=0.20$), and therefore, males and females could not be distinguished on the basis of their alkaloid composition. However, although overall alkaloid composition was not significantly different between sexes, there do appear to be certain alkaloids that are related to sex in *M. bernhardi*. These alkaloids include the 1,4-disubstituted quinolizidine **231A** (present in six males in minor amounts, but in only one female and only in a trace amount), the 3,5-disubstituted indolizidine **249A** (present in 11 females, but in only 4 males), and the 3,5-disubstituted indolizidine **275C** (present in six females, but in only one male). The presence of **249A** and **275C** (mainly in females) is presumably because of sequestration from myrmicine ants, whereas the presence of **231A** (mainly in males) presumably is because of sequestration from oribatid mites. The differences in the presence of certain alkaloids observed between sexes of *M. bernhardi* may be explained by differences in diet (preference or availability based on behavior) between sexes. Breeding of this species occurs in swamps or near ponds in the forest, where males call from particular positions on the ground, close to the water, probably delimiting at least short-term territories. Consequently, females probably have larger home ranges than males, at least during the reproductive season, and are therefore presumably encountering a more diverse array of alkaloid-containing arthropods, whereas males are more subject to local availability of alkaloid-containing arthropods. In addition, female *Mantella* frogs are typically larger (SVL) than male frogs, which may suggest that they would consume more prey and/or have a larger capacity for storage of alkaloids. The tendency for larger females is particularly clear in *M. bernhardi*, where in the populations from Manombo and Vevembe, the males can be quite small (indeed being the smallest adult *Mantella* known) and the females distinctly larger (males 14–18 vs. females 17–20 mm SVL). In *M. bernhardi* from Manombo (Table 5), none of the 11 males had total alkaloid amounts classified as major (+++), whereas 11 of the 14 females did. However, the importance of location with respect to total amount of alkaloids is clear from a comparison to the *M. bernhardi* from Vevembe (Table 6), where of the 25 frogs (mostly males), all but one male and one female had alkaloid amounts classified as major (+++). Unfortunately, nothing is known as to whether any of the mantellid frogs have reached the maximum alkaloid storage capacity of the cutaneous (poison) glands. Furthermore, it should be noted that any general conclusions as to sex differences should be treated as tentative, because of the preponderance of males in all collections except *M. bernhardi* from Manombo, in which differences between sexes were detected with respect to certain alkaloids and perhaps with respect to amounts.

Poison glands are known to increase in diameter with increases in size of a poison frog, as has been shown for the dendrobatid poison frog *Oophaga pumilio* (Saporito et al., unpublished data), suggesting that larger frogs might have a larger storage capacity for alkaloids. The total amount of alkaloids is one measure of capacity, whereas the total number of alkaloids present in an individual frog can be thought of as a rough measure of the diversity of alkaloid-containing arthropods consumed by an individual over a lifetime. Within a species, larger poison frogs can be presumed to be older than smaller frogs. On the basis of these presumptions, it might be expected that the number of alkaloids (diversity) would be greater in larger mantellid frogs, both within and among species. When the three mantellid species were examined together, the number of alkaloids (including trace alkaloids) was positively correlated with frog size (Fig. 6; $P<0.001$; $R^2=0.37$), and in general, larger frog species tended to have a larger number of alkaloids. Clark et al. (2006) also reported a positive relationship between frog size (*M. baroni* vs. *M. bernhardi*) and the total number of alkaloids. However, in the present study, when each mantellid species is examined separately, size is related to the number of alkaloids only in *M. baroni* (Fig. 6; $P=0.002$; $R^2=0.27$). The lack of a strong relationship between frog size and the number of alkaloids within a mantellid species suggests that frog size (and probably age) is not the main determinant of total alkaloid number (alkaloid diversity). However, the differences between species may be at least partly explained by their different sizes and ages. Data on the age of specimens in

wild *Mantella* populations are needed to test this hypothesis. Inclusion of additional species and increased sample sizes are necessary to determine the extent by which size influences the diversity of alkaloids within a mantellid species. Analyses of alkaloids in individuals of two relatively small mantellid frogs, namely, *M. aurantiaca* and *M. milotympanum* are in progress.

The report by Clark et al. (2006) proposed that mantellid frogs from relatively pristine sites have a greater number of alkaloids than those from disturbed sites, suggesting that levels of disturbance are directly related to alkaloid diversity in poison frogs. Although differences in disturbance likely play a role in the abundance and distribution of alkaloid-containing arthropods, which likely influences alkaloid compositions in frogs, we do not think that the data presented in Clark et al. (2006) are compelling enough to make the claim that disturbance negatively influences alkaloid diversity in poison frogs. The proposal by Clark et al. (2006) is based on a comparison of the total number of alkaloids among very limited numbers of individuals of *M. baroni* from three different geographic locations (ranked as pristine, slightly disturbed, and disturbed). Given the amount of alkaloid variation within populations of poison frogs (illustrated in this study; also see Saporito et al. 2006), it is not clear what conclusions can accurately be made with such small sample sizes. In addition, Clark et al. 2006 were not justified in including four individuals from a disturbed site in which alkaloids were not obtained by extraction of the skin but solely by electrical stimulation of the skin (transcutaneous amphibian stimulation). Other critical comments on the data of Clark et al. (2006) are included in the present Supplementary Information.

Alkaloid compositions for *M. baroni* from seven sites, some relatively undisturbed and some disturbed, had been previously reported (Daly et al. 1996). A discussion of that study was neglected by Clark et al. (2006). The *M. baroni* from four disturbed areas had an average of 20 alkaloids per site, whereas those from three relatively undisturbed areas had an average of 17 alkaloids per site (Daly et al. 1996). Analyses were of combined skin samples from each site. In addition, the gas chromatograms presented in Daly et al. (1996) and Garraffo et al. (1993a) do not show any clear relationship between the amounts of alkaloids for *M. baroni* and the disturbance of a collection site. Of the four disturbed sites, two show major amounts of alkaloids, and two show minor amounts. Of the three relatively undisturbed sites, one shows major amounts, the other two, minor (Garraffo et al. 1993a; Daly et al. 1996) Thus, neither the number of alkaloids nor the amounts of alkaloids appeared to correlate with the degree of disturbance at a location.

The present alkaloid analyses of *M. baroni* also do not support the conclusion that frogs from an undisturbed collection site will have a larger number or a greater diversity of alkaloids. However, it should be noted that sample sizes in the present study also were small and, therefore, any conclusion should be treated as tentative. For the sites of the five combined skin collections of *M. baroni*, Mangevo and Vohiparara in the Ranomafana National Park are considered relatively undisturbed sites, whereas Andriabe, Tsinjoarivo, and Vohindrazana are considered disturbed sites (see Table 7). The two relatively undisturbed sites have, respectively, a total of 17 alkaloids (of which ten are major/minor) and 19 alkaloids (of which four are major/minor). The total number of alkaloids for the disturbed sites, in the order listed above, is as follows: 24 alkaloids (of which 17 are major/minor), 22 alkaloids (of which 12 are major/minor); and 30 alkaloids (of which 12 are major/minor). A comparison of the number of alkaloids in *M. baroni* between these sites clearly demonstrates that there are not significantly more alkaloids in frogs from the undisturbed sites. Furthermore, in the present study, the 15 individual skins of *M. baroni* from Ranomafana (an undisturbed site) contained an average of 7.0 major/minor alkaloids per frog (average including trace alkaloids, 16), whereas the four individuals from Besariaka (a disturbed site) contained an average of 8.3 major/minor alkaloids (average including trace alkaloids, 13). Once again, there is no marked difference in the number of alkaloids (a measure of alkaloid diversity) between an undisturbed and a disturbed site for *M. baroni*. At the present time, the generalization by Clark et al. (2006) that a pristine site will yield frogs with either a greater diversity or amount of alkaloids cannot be supported with the available data.

The current study resulted in the detection of 46 alkaloids (including isomers) in 19 individuals of *M. baroni* from two different populations, 56 alkaloids (including isomers) in 11 individuals of *M. madagascariensis* from two different populations, and 48 alkaloids (including isomers) in 51 individuals of *M. bernhardi* from two different populations (Tables 1, 2, 3, 4, 5, and 6). Summaries of alkaloid composition of extracts of individuals from six populations (Table 7) and of extracts from combined skins (Table 11) indicate the marked dependence of alkaloid compositions on the geographic location of the collections.

A variety of factors undoubtedly affect the complex differences in alkaloid composition detected in mantellid frogs. The geographic location of mantellid species and associated differences in habitat between locations, as well as the availability of alkaloid-containing arthropod prey items within each habitat are likely the most important factors in explaining variation in alkaloid composition. Temporal differences in alkaloid composition are likely because of successional changes in habitat and the associated shifts in alkaloid-containing prey availability. In addition, difference in prey electivity and/or foraging

behavior, which may be correlated in some cases with certain species, sexes, age, and/or size of mantellids, may also play a role in mantellid alkaloid variation. Finally, it is possible that some of the variation in alkaloid composition is because of differences in the alkaloid uptake systems among different species, which are presumably involved in sequestration and retention of alkaloids. A simplistic analysis of the factors involved in explaining variation in alkaloid composition is clearly not possible, but the various confounding factors are amenable to further study.

There is currently no information on whether or not there is a preference for sequestration and/or storage of one alkaloid structural class over another in mantellid frogs. A series of controlled and extensive feeding experiments will be required to clarify these points. However, as yet, the resources, which include a large number of captive-raised frogs, have not been available. Several of the mantellid species are now at risk (Andreone et al. 2005; Vieites et al. 2006), which may preclude extensive studies of mantellids similar to the present study. The wide range of amounts of alkaloids present in individual frogs from a single collection site is perhaps the most important current observation and has required these extensive single skin analyses to verify. The rare complete absence of alkaloids or the presence of only a trace amount of alkaloids in individual frogs is somewhat puzzling. It is possible that such frogs have expelled most of their alkaloids just before capture; however, it is unlikely that all of the alkaloids are expelled as a defensive strategy. Therefore, a deficient or possibly absent uptake system for these rare individuals is the most likely explanation at the moment. Those frogs without alkaloids or with only trace amounts of alkaloids appear to occur randomly in every collection.

Further research is clearly needed to understand the complex trophic relationships between alkaloid-containing mites, ants, millipedes, and beetles and chemical defense in poison frogs. Crucial to such an understanding will be (1) the distribution and composition of alkaloids within oribatid mites and myrmicine ants, which appear likely to be the principal dietary sources of poison frog alkaloids, (2) the factors that affect abundance/availability of such prey, and (3) the electivity of frogs toward such alkaloid-containing prey. Multiple classes of alkaloids do occur together in mites, but can also occur alone (Saporito et al. 2007b). In ants, alkaloid composition differs not only with species, but also with caste and age (Deslippe and Guo 2000; Torres et al. 2001; Saporito et al. 2004). Studies of alkaloid composition in different species of Madagascan mites, the occurrence of such mites in stomach contents of mantellid frogs, and correlations between alkaloids of mites, ants, and frogs from the same site are required.

Analyses of alkaloid profiles in more than 80 individual frogs of the *M. milotympanum* group (Vences et al. 1999), namely, *M. aurantiaca*, *M. milotympanum*, and *M. crocea*, are in progress (Daly and Vences et al., unpublished data). In parallel studies, alkaloid profiles have been obtained for extracts of combined frogs of 13 species of *Mantella* from more than 40 different sites in Madagascar (N. R. Andriamaharavo, M. Andriantsiferana et al., unpublished data), and the profiles are being analyzed for further insights into the factors that determine alkaloid compositions in these poison frogs.

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