Formicine ants: An arthropod source for the pumiliotoxin alkaloids of dendrobatid poison frogs

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A remarkable diversity of bioactive lipophilic alkaloids is present in the skin of poison frogs and toads worldwide. Originally discovered in neotropical dendrobatid frogs, these alkaloids are now known from mantellid frogs of Madagascar, certain myobatrachid frogs of Australia, and certain bufonid toads of South America. Presumably serving as a passive chemical defense, these alkaloids appear to be sequestered from a variety of alkaloid-containing arthropods. The pumiliotoxins represent a major, widespread, group of alkaloids that are found in virtually all anurans that are chemically defended by the presence of lipophilic alkaloids. Identifying an arthropod source for these alkaloids has been a considerable challenge for chemical ecologists. However, an extensive collection of neotropical forest arthropods has now revealed a putative arthropod source of the pumiliotoxins. Here we report on the presence of pumiliotoxins in formicine ants of the genera *Brachymyrmex* and *Paratrechina*, as well as the presence of these ants in the stomach contents of the microsympatric pumiliotoxin-containing dendrobatid frog, *Dendrobates pumilio*. These pumiliotoxins are major alkaloids in *D. pumilio*, and *Brachymyrmex* and *Paratrechina* ants now represent the only known dietary sources of these toxic alkaloids. These findings further support the significance of ant-specialization and alkaloid sequestration in the evolution of bright warning coloration in poison frogs and toads.

allomones | allopumiliotoxins | cardiotonic activity | chemical defense | myrmicine ants

More than 500 alkaloids have been detected in skin extracts of anurans, and most of these have been assigned to 24 different structural classes (1). Presumably serving as a passive chemical defense against predation and/or microorganisms, these alkaloids originally were thought to be a product of anuran metabolism (2). Evidence now indicates that poison frogs merely have an efficient system that accumulates alkaloids from dietary alkaloid-containing arthropods (3–8). Dendrobatid (*Dendrobates*, *Epipedobates*, and *Phyllobates*) and mantellid (*Mantella*) frogs raised in captivity do not have detectable alkaloids, yet they possess the ability to selectively accumulate alkaloids provided to them through diet (8, 9). It seems likely that the bufonids (*Melanophryniscus*) will also have a similar dietary uptake system (10). Myobatrachid (*Pseudophryne*) frogs of Australia appear to synthesize their indolic pseudophrynamine alkaloids but sequester pumiliotoxins from a dietary source (11).

Representatives of many of the structural classes of alkaloids found in poison frogs and toads are known to occur in specific arthropods and thus such arthropods represent likely dietary sources. Some of these alkaloids, shared by arthropods and poison frogs and toads, are depicted in Fig. 1. Ants of the subfamily Myrmicinae appear to be the source for the 2,5-disubstituted pyrrolidines, the 2,6-disubstituted piperidines, the 3,5-disubstituted pyrrolizidines, the 3,5-disubstituted indolizidines, the 4,6-disubstituted quinolizidines, and the 2,5-disubstituted decahydroquinolines (3, 4). The 3,5-disubstituted lehmizidines (12), the histrionicotoxins (1), and the gephyrotoxins (1) represent alkaloid classes (Fig. 2) that share certain structural features with those of known ant alkaloids, and it is expected that they will prove to be of myrmicine ant origin as well. All of the alkaloids in frog and toad skin that appear to originate by sequestration from myrmicine ants contain unbranched carbon skeletons. Coccinellid beetles appear to be a dietary source for the coccinellines and some of the structurally related tricyclic alkaloids (5). Siphonotid millipedes are the putative dietary source for the spiropyrrolizidine oximes and nitropolyzonamines (7). Although several classes of frog skin alkaloids have been identified in arthropods, certain major structural classes (e.g., the steroidal batrachotoxins, the cardiotonic pumiliotoxins, and the analgetic epibatidine) have not yet been identified from a specific arthropod source. A pumiliotoxin and an allopumiliotoxin were detected in mixed collections of small arthropods from Panama (6). Such alkaloids have a branched carbon skeleton with apparent isoprenoid moieties and therefore were not expected to be of myrmicine ant origin.

In an attempt to identify an arthropod source for the pumiliotoxin family of alkaloids, we made several extensive arthropod collections on Isla Bastimentos, Bocas del Toro Province, Panama. This area was chosen based on the high levels of pumiliotoxins A and B (**307A** and **323A**, respectively; Fig. 3) present in populations of the dendrobatid frog *Dendrobates pumilio*, and on the previous detection of **307A** in mixed leaf-litter collections of arthropods from the same location (6). The pumiliotoxins and most other alkaloids from anuran skin have been assigned code names, which consist of a boldfaced number corresponding to the nominal mass and a boldfaced letter for identification of individual alkaloids (1).

Materials and Methods

Arthropod Collections. We collected arthropods from the following locations on Isla Bastimentos during the dry and wet seasons (February 2–8 and August 20–23, 2003, respectively). Site 1: northwest coast, secondary forest; leaf litter, abundant; 9°21.618′N, 82°12.074′W. Site 2: northwest coast, numerous *Cyclanthus* and cacao; leaf litter, abundant; 9°21.250′N, 82°12.519′W. Site 3: northwest coast, numerous *Heliconia*; leaf litter, sparse; 9°21.169′N, 82°12.627′W. Site 4: northwest coast, numerous *Heliconia*; leaf litter, abundant; 9°21.123′N, 82°12.620′W. Site 5: northwest coast, numerous palms; leaf litter, abundant; 9°20.996′N, 82°12.726′W. Site 6: south coast, secondary forest; leaf litter, abundant; 9°20.364′N, 82°10.807′W. Site 7: northwest coast, numerous *Cyclanthus*; leaf litter, abundant; 9°21.021′N, 82°12.704′W. Site 8: inland, secondary forest; leaf litter, abundant; 9°20.490′N, 82°10.486′W. All site descriptions are based on the dominant plant type(s) or forest type at each site. The amount of leaf litter at each site was ranked as either

See Commentary on page 7841.

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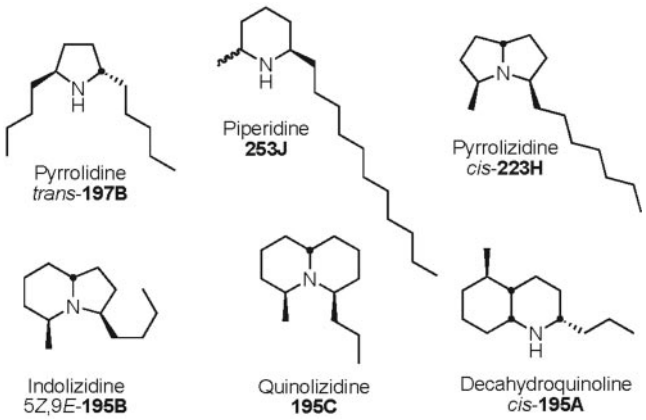

Fig. 1. Structures of representative alkaloids common to myrmicine ants and poison frogs and toads.

sparse or abundant. Mixed arthropod collections were made at sites 1–3, 5, and 6 previously (6).

We collected all arthropod samples with forceps, from leaf litter (6, 7) or directly from vegetation, and placed them in taxon-specific 1.5-ml plastic vials containing methanol. The forceps were cleaned with methanol between each sampling event. Only small arthropods (<10 mm), suitable as prey for *D. pumilio*, were collected from these sites. After arthropod collections, we collected five frogs from each site during both seasons for analysis of skin alkaloids. We also stomach-flushed 180 frogs during each season to obtain dietary information from four sites (1, 3, 5, and 6). All voucher specimens are located in the herpetological collection at Florida International University.

Analysis of Alkaloid Extracts. Methanol extracts of arthropods were analyzed by using gas chromatography mass spectral analysis (GC-MS), conducted with a Finnigan GCQ instrument with a 30 m × 0.25 mm i.d. DB-1 fused silica column with a temperature program from 100 to 280°C at a rate of 10°C/min. All ant extracts were concentrated to a volume of 10 μl before analysis. The identification of **307A** and **323A** was based on the comparison of GC mass spectra to authentic standard samples of these compounds.

Methanolic alkaloid fractions were prepared and partitioned for all frog skins as described in ref. 3. Major and minor alkaloids were identified by using a flame-ionization detector after GC on a 6-foot 1.5% OV-1 packed column (2 mm i.d.), programmed from 150 to 280°C at a rate of 10°C/min. An injection volume of 2 μl was used for each frog alkaloid fraction, corresponding to 2 mg of wet weight frog skin (3).

Results

GC-MS analyses of >500 arthropod samples (255 samples during the dry season and 257 samples during the wet season) led to the detection of **307A** and **323A** in formicine ants of the genera *Brachymyrmex* (*Brachymyrmex longicornis* and *Brachymyrmex* cf. *depilis*; Fig. 4*A*) and *Paratrechina* (*Paratrechina steinheili*; Fig. 4*B*), representing two different ant tribes, Myrmelachistiani and Prenolepidini, respectively. With the exception of pyrazines, which are widespread as pheromones in the family Formicidae (13), alkaloids have never been detected in ants of the subfamily Formicinae, which are characterized by the presence of formic acid in poison glands (14). Pyrazines were not detected in either *Brachymyrmex* or *Paratrechina* extracts, but were detected in other ant extracts. During the wet season, all *Brachymyrmex* samples were collected from plants of the genus *Heliconia*. Pumiliotoxins **307A** and **323A** were detected in *Brachymyrmex* samples from sites 5 and 7, whereas only **307A** was detected in *Brachymyrmex* samples from sites 3, 4, and 8. Not all *Brachymyrmex* samples collected from these sites contained pumiliotoxins. *Brachymyrmex* also were collected from site 1; however, **307A** and **323A** were not detected. During the dry season, *Brachymyrmex* were only collected from site 7 from the leaf litter and no pumiliotoxins were detected. Pumiliotoxins **307A** and **323A** were detected in *P. steinheili* samples collected from the leaf litter in site 5 during the wet season. *Brachymyrmex* cf. *depilis* was identified in the stomach contents of three frogs from site 6 and one frog from site 5, and *P. steinheili* was identified in the stomach contents of one frog from site 5. Pumiliotoxins **307A** and **323A** were detected in skin extracts of frogs from the sites at which the pumiliotoxins were detected in ants (data not shown). The presence of **307A** and **323A** in ants of the genera *Brachymyrmex* and *Paratrechina* as well as in skin extracts of the dendrobatid frog *D. pumilio*, coupled with the presence of these ants in stomach contents of *D. pumilio*, strongly suggests that these ants represent a dietary source for pumiliotoxins in these populations of frogs. Pumiliotoxins **307A** and **323A** were not detected in any of the other several hundred arthropod extracts examined in this study.

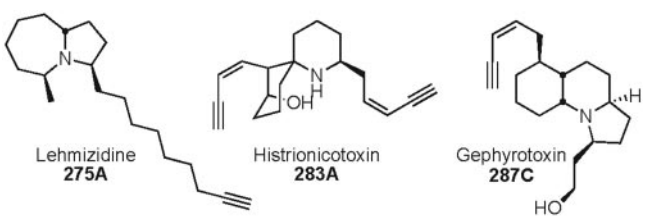

Fig. 2. Structures of alkaloids from dendrobatid poison frogs suspected to be of myrmicine ant origin.

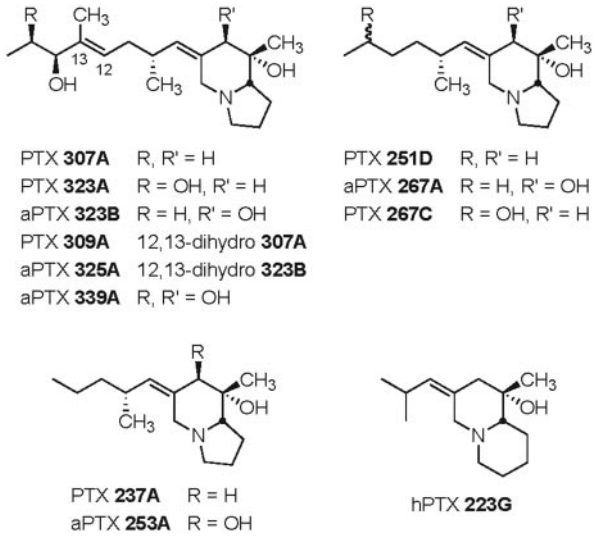

Fig. 3. Structures of pumiliotoxins A (**307A**) and B (**323A**) and other pumiliotoxins (PTX), allopumiliotoxins (aPTX), and a homopumiliotoxin (hPTX) found as major/minor alkaloids in skin extracts of poison frogs.

Discussion

The pumiliotoxin family of alkaloids consists of >80 alkaloids, which are divided into three major classes: the pumiliotoxins, the allopumiliotoxins, and the homopumiliotoxins (1). The pumiliotoxins and allopumiliotoxins are found in all anuran genera known to contain lipophilic alkaloids, which include frogs and

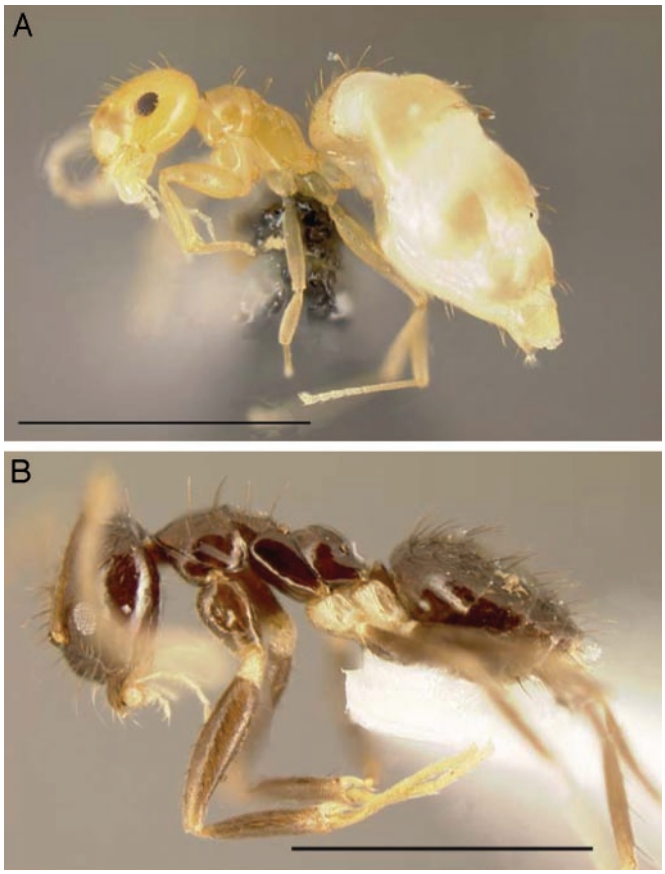

Fig. 4. Pumiliotoxin-containing ants from Isla Bastimentos, Bocas del Toro, Panama. (*A*) *B. longicornis*. (*B*) *P. steinheili*. (Bar, 1 mm.) The images were created by J.T.L.

toads from a variety of locations worldwide (2, 10, 11, 15). Therefore, it is expected that the dietary source(s) of the pumiliotoxins will share a similar distribution. Pumiliotoxins **307A** and **323A** occur frequently as major and minor alkaloids in dendrobatid frogs of the genus *Dendrobates*, occur less frequently in *Minyobates*, and are absent in *Epipedobates* and *Phyllobates* (Table 1). They also occur as major or minor alkaloids in frogs of the genus *Mantella*, although a dihydroanalog (**309A**; Fig. 3) is more common (Table 2). Neither **307A** nor **323A** occur as major or minor alkaloids in toads of the genus *Melanophryniscus*, where a simpler alkaloid, pumiliotoxin **251D**, is often a major alkaloid (Table 2). Pumiliotoxin **323A**, but not **307A**, occurs in frogs of the genus *Pseudophryne*, where pumiliotoxin **267C** (Fig. 3) and allopumiliotoxin **323B** are the only other alkaloids of the pumiliotoxin family to be detected as major or minor alkaloids (Table 2). Pumiliotoxin **267C** occurs only rarely in neotropical frogs of the family Dendrobatidae (Table 1). Other members of the pumiliotoxin family, most commonly **251D** and **267A** (Fig. 3), are present as major or minor alkaloids in all genera of dendrobatids, except *Phyllobates* (Table 1). Of the two, only **251D** occurs in the genera *Mantella* and *Melanophryniscus*. Neither **251D** nor **267A** occurs in *Pseudophryne*. Some of the allopumiliotoxin **267A** found in dendrobatid extracts may have been formed by hydroxylation of dietary pumiliotoxin **251D**, as has been shown specifically for frogs of the genus *Dendrobates* (19).

The genus *Brachymyrmex* is endemic to the American tropics and subtropics, with ≈40 described species (20, 21). A few species are found in the Old World and are generally restricted to synanthropic habitats. The genus *Paratrechina* is found in the tropics and subtropics throughout the world (22). Ants of the genus *Paratrechina* are very common in leaf litter of lowland wet forests, and *P. steinheili* is one of the most abundant ant species in the neotropics. Therefore, ants in the genera *Brachymyrmex* and *Paratrechina* are expected to be coextensive with pumiliotoxin-containing dendrobatid frogs and bufonid (*Melanophryniscus*) toads (see Tables 1 and 2 for distribution of pumiliotoxins in dendrobatid frogs and bufonid toads, respectively), and it seems probable that species in these ant genera serve as a source for the pumiliotoxins. Ants in the genus *Paratrechina* may serve as a source for pumiliotoxins in Madagascan mantellid (*Mantella*) frogs and Australian myobatrachid (*Pseudophryne*) frogs (see Table 2 for distribution of pumiliotoxins in Old World frogs).

Not all of the *Brachymyrmex* samples examined in this study contained pumiliotoxins. Pumiliotoxins were found in extracts from five of the six sites in which *Brachymyrmex* were collected but not in all *Brachymyrmex* samples from these sites. These data suggest that there is both spatial and temporal variation in the presence of pumiliotoxins among *Brachymyrmex* ants. Caste-specific alkaloid production is known among myrmicine ants of the genus *Solenopsis* and may occur in ants of the genus *Brachymyrmex* (23, 24).

Ants of the subfamily Formicinae are well known for the use of formic acid as a chemical defense against predation. Alkaloids other than the widely distributed pyrazines, which serve as pheromones in many ants (13), have never been detected in ants of the subfamily Formicinae. Pumiliotoxins and allopumiliotoxins are highly toxic (6, 25) and thus presumably also serve as a chemical defense in *Brachymyrmex* and *Paratrechina*, along with formic acid. Pumiliotoxins are potent cardiotonics (26, 27) and are therefore of some interest as pharmacological probes (28, 29) and even as potential therapeutics. Certain pumiliotoxins are potent insecticides (30). The present discovery of an ant source of the pumiliotoxins may permit the identification of the gene cluster involved in synthesis of these structurally unique alkaloids.

Despite the large number and diversity of alkaloids previously isolated from myrmicine and pseudomyrmicine ants (13), only two reports document the biosynthesis of alkaloids in such ants (31, 32). Alkaloid sequestration from plants has been well documented among Lepidopteran and Coleopteran arthropods (33) but has not been reported for ants. A role for a microsymbiont in production of ant alkaloids cannot be precluded.

Dendrobatid frogs of the genera *Dendrobates*, *Epipedobates*, and *Phyllobates* consume a high proportion of ants as part of their diet in the wild (34–36). The term "ant-specialist" has been used to describe frogs in the genus *Dendrobates* and some members of the genus *Epipedobates* (*Epipedobates trivittatus*, *Epipedobates petersi*, and *Epipedobates pictus*), based on the fact that they consume ants in higher proportions than they are available in their environment (35, 36). However, only *Epipedobates erythromos*, *Epipedobates espinosai*, *Epipedobates silverstonei*, and *Epipedobates tricolor* of 11 *Epipedobates* species contained minor or major amounts of pumiliotoxins (Table 1). Most, including *E. trivittatus*, *E. petersi*, and *E. pictus*, contained mainly decahydroquinolines and histrionicotoxins (2), presumably sequestered from dietary myrmicine ants. It should be noted that alkaloids of the pumiliotoxin class (Fig. 3) are much more toxic than the "izidine" alkaloids, decahydroquinolines, and histrionicotoxins (Figs. 1 and 2). Although not as extensively studied, alkaloid-containing frogs in non-dendrobatid genera (*Mantella*, *Melanophryniscus*, and *Pseudophryne*) have also been shown to consume large numbers of ants (37–39). Currently, 7 of the 24 structural classes of anuran alkaloids are known to also occur in ant extracts. An additional 8 alkaloid classes now are suspected to be of ant origin. Thus, myrmecophagy in dendrobatid frogs

Table 1. Occurrence of pumiliotoxins (PTX), allopumiliotoxins (aPTX), and a homopumiliotoxin (hPTX) as major and minor alkaloids in extracts from dendrobatid frogs

Genus and species	Location	Year	Pumiliotoxins														
			hPTX 223G	PTX 237A	PTX 251D	aPTX 253A	aPTX 267A	PTX 267C	PTX 281A	aPTX 297A	PTX 307A	PTX 309A	PTX 323A	aPTX 323B	aPTX 325A	aPTX 339A	aPTX 341A
Dendrobates																	
arboreus	Chiriquí, Panama	1983			○		○				○		●				
auratus	Isla Taboga, Panama	1974			○		○						○	○			
	El Cope, Coclé, Panama	1977	○				●				○		●	○			
	Isla Pastores, Bocas, Panama	1982					○						○				
	Cerro Ancón, Panama	1992									●		○	○		○	
	Manoa Valley, Oahu, Hawaii	1988			●		●	○					○				
azureus	Sipaliwini, Surinam	1972					○										
granuliferus	Puntarenas, Costa Rica	1967					○				○		●				
histrionicus	Altos de Buey, Chocó, Colombia	1978			○		●						○				
lehmanni	Río Anchicaya, Valle, Colombia	1973				○	●				○		●				○
leucomelas	Represa Guri, Bolívar, Venezuela	1968					●						●	○			
occultator	Río Saija, Cauca, Colombia	1973											●				
pumilio	Bastimentos, Isla Bastimentos, Bocas, Panama	1972					○				●		●	○			
	Bastimentos, Isla Bastimentos, Bocas, Panama	1981, 2000									●		●	○			
	Sol Creek, Isla Bastimentos, Bocas, Panama	1984					○				●			○			
	Isla Pastores, Bocas, Panama	1981			○		●										
	Mainland, NW Isla Split Hill, Bocas, Panama	1981									●		○				
	Isla Cayo Agua, Bocas, Panama	1981			●		●				○		○	○			
	W. Rumpala, Bocas, Panama	1981			●		○										
	Isla Popa (North), Bocas, Panama	1981			●		○										
	Almirante, Bocas, Panama	1983			●		○										
	Siquierres, Limón, Costa Rica	1995									●		○		○		
quinquevittatus	Rondonia, Brazil	1985					○							●			
reticulatus	Loreto, Peru	1977					●				○						
speciosus	Que. de Arena, Chiriquí, Panama	1983			○		●				●		●				
	Que. de Frank, Chiriquí, Panama	1983			●		●				○		○				
tinctorius	Coppename River, Surinam	1973					●										
ventrimaculatus	Pebas, Loreto, Peru	1977		○		●	○										
	Mishana, Loreto, Peru	1977		○	○	○	●										
vicentei	El Cope, Coclé, Panama	1977			○	○	○				●		○				
Epipedobates																	
erythromos	Río Palenque, Pichincha, Ecuador	1979					○										
espinosai	Río Palenque, Pichincha, Ecuador	1979		○													
silverstonei	Huánuco, Peru	1974			○									○			
tricolor	Santa Isabel, Azuay, Ecuador	1977			●		○							○			○
Minyobates																	
abditus	Napo, Ecuador	1974		●		○							○	○			
altobueyensis	Altos de Buey, Chocó, Colombia	1978					●				○	○			●		
bombetes	Lago de Calima, Valle, Colombia	1976			●								○	○			
claudiae	Isla Colon, Bocas, Panama	1977			○		○		○	○							
	New Guinea, Isla Bastimentos, Bocas, Panama	1986					●						○		○		
*fulguritus**	El Llano-Carte Rd, Panama, Panama	1974					○				○		○				
minutus†	Cerro Campana, Panama, Panama	1972					●		○	○							
	Altos de Buey, Chocó, Colombia	1978			●		●				○	●			●		○
	Río Saija, Cauca, Colombia	1978					●				○	●	○		●		
steyermarki	Cerro Yapacana, Venezuela	1978				○	●										
viridis	Río Clara, Valle, Colombia	1983			○		●					○	○				○

●, Major (>50 μg per 100 mg of skin); ○, minor (>5 μg per 100 mg of skin). Only major and minor alkaloids from each species/population are tabulated. Other extracts containing only minor or trace amounts of pumiliotoxins are not reported. The generic classifications are those of Myers (16). For structures see Fig. 3 and references. Data are from ref. 2 and unpublished results.

**Dendrobates fulguritus* sensu Vences *et al.* (17).

†*Dendrobates minutus* sensu Clough and Summers (18).

has likely played a major role in the evolution of alkaloid sequestration and aposematism (35–37, 40, 41). Recent evidence supporting at least two origins of "diet specialization" and aposematism in dendrobatids appears to support this claim (41). The discovery of pumiliotoxins in formicine ants provides further evidence for the importance of myrmecophagy and alkaloid

Table 2. Occurrence of pumiliotoxins (PTX), allopumiliotoxins (aPTX), and a homopumiliotoxin (hPTX) as major and minor alkaloids in extracts from bufonid, mantelid, and myobatrachid poison frog

Family, genus, and species	Location	Year	Pumiliotoxins										
			hPTX 223G	PTX 251D	aPTX 267A	PTX 267C	PTX 307A	PTX 307F	PTX 307G	PTX 309A	PTX 323A	aPTX 323B	aPTX 325A
Bufonidae													
Melanophryniscus	South America												
montavidarais	La Coronilla, Roche, Uraguay	1987		●									
moraires	Serra de Mantiguenra, Rio de Janeiro, Brazil	1979				●						○	
emsstalanari	Tanti, Córdoba, Argentina	1989		●									
Mantellidae													
Mantella	Madagascar												
aurantiaca	N. Andasibe	1989				○	○				●	○	
baroni	N. Andasibe	1989					○	○		●			○
	E. Andasibe	1993						○	○	●			○
	S. Moramango	1989					○			○			●
	An' Ala	1993						○		●	○		
	Ranomafana	1993						○		●	○		
	Sahavondrona	1993		○				○		●			
betsileo	Farakaraina	1993	○	○					●				
cowani	Antoetra	1993		●		○			○				
crocea	N. Andasibe	1989				●	●				○	●	
expectata	Isalo	1993		●			○						
laevigata	Nosy Mangabe	1993							●				
pulchra	Ambavala	1990				○							
	An' Ala	1993		○			○	○		●		○	○
viridis	Montagne des Francais	1994					●				○		
Myobatrachidae													
Pseudophryne	Australia												
australis	Pearl Beach, New South Wales	1989									●		
bibroni	Nortin Summit, South Australia	2000				●							
coriacea	Daisy Hill, Queensland	1987										●	
corroboree	Round Mtn, New South Wales	1989				○					●	○	
pengilley	Yarrangobilly, New South Wales	1987				○					○		
semimarmorata	Holy Plains State Park, Victoria	1999				●							

●, Major (>50 μg per 100 mg of skin); ○, minor (>5 μg per 100 mg of skin). Only major and minor alkaloids from each species/population are tabulated. Other extracts containing only minor or trace amounts of pumiliotoxins are not reported. For structures see Fig. 3 and references. Data are from refs. 10, 11, and 15.

sequestration in the evolution of aposematic coloration in poison frogs and toads.

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