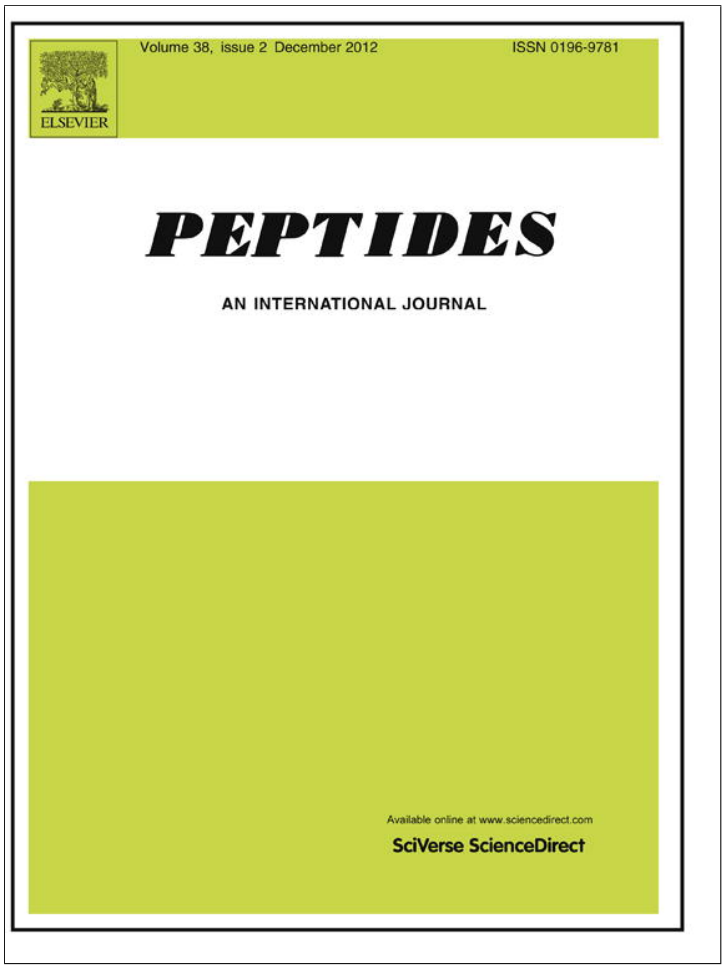

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Molecular cloning of skin peptide precursor-encoding cDNAs from tibial gland secretion of the Giant Monkey Frog, *Phyllomedusa bicolor* (Hylidae, Anura)

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ABSTRACT

The skins of phyllomedusine frogs have long been considered as being tremendously rich sources of bioactive peptides. Previous studies of both peptides and cloning of their precursor encoding cDNAs have relied upon methanolic skin extracts or the dissected skins of recently deceased specimens and have not considered the different glands in isolation. We therefore focused our attention on the tibial gland of the Giant Monkey Frog, *Phyllomedusa bicolor* and constructed a cDNA library from the skin secretion that was obtained via mechanical stimulation of this macrogland. Using shotgun cloning, four precursors encoding host-defense peptides were identified: two archetypal dermaseptins, a phyllokinin and a phylloseptin that is new for this species but has been recently described from the Waxy Monkey Leaf Frog, *Phyllomedusa sauvagii*. Our study is the first to report defensive peptides specifically isolated from anuran tibial glands, confirming the hypothesis that these glands also contribute to chemical defense. Moreover, the discovery of novel compounds for this otherwise very well characterized species suggests that this largely neglected gland might possess a different cocktail of secretions from glands elsewhere in the same animal. We will also discuss some evolutionary implications of our findings with respect to the adaptive plasticity of secretory glands.

1. Introduction

Amphibians have a highly specialized skin that is rich in both granular and mucous glands, the latter helping to maintain a moist skin in a terrestrial environment to enable key physiological functions such as cutaneous respiration [25]. By contrast, granular (or serous) glands contain a wide range of bioactive molecules that serve defensive roles. These compounds include alkaloids, quinones, steroids, biogenic amines and a remarkable diversity of biologically active peptides [16,22,26]. Phyllomedusine frog skin secretions are particularly interesting due to the presence of vasoactive peptides [27]. For example, skin secretions of the Giant Monkey Frog (*Phyllomedusa bicolor*) are used in the extraordinary hunting ceremonies of the Matses Indians in Peru [21]. The biological manifestations caused by skin secretions applied during these ceremonies (e.g., increased stamina, satiety) have prompted extensive pharmacological studies of the skin extracts [28] as well as promoting sampling of related frog species. The end result of all these activities confirmed the recognition that the integument of amphibians in general, and not only of phyllomedusine frogs, is a "huge factory and store-house" of bioactive compounds [29], among which peptides constitute the major components and that these compounds are of particular interest as potential sources for clinical therapeutics [5,19].

For phyllomedusine frogs, the complex mixture of defensive peptides in their skin secretion includes tachykinins, bombesin-like peptides, bradykinin-related peptides (BRPs), caeruleins, phyllokinins, opioids/dermorphins [34] and sauvagines [43]. The latter two peptide families occur exclusively in this subfamily, whereas the others also occur in other anuran amphibians (Table 1). As for most frog species, the defensive peptides in phyllomedusines co-occur with broad-spectrum antimicrobial peptides (AMPs), the largest and most structurally diverse peptide family known from frog skin secretions and which in phyllomedusines includes dermaseptins, phylloseptins, plasticins, dermatoxins, phylloxins and hyposins [2,7].

Most recorded phyllomedusine skin peptides have been discovered from either pooled methanolic skin extracts or by dissection of recently deceased specimens [27], with DNA isolation from blood having been used to identify the dermaseptin gene structure [30,51]. In addition, the gland contents have typically been pooled following acquisition of secretion and hence represent a

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Table 1
Overview of peptide families isolated from frog skin.

Source/taxon	AMPs	BLP	BRP	Cae	Tachy	Tryp	Op*	Sau*
Ascaphidae	+		+			+		
Bombinatoridae	+	+	+					
Alytidae	+	+						
Pipidae	+			+				
Heleophrynidae			+					
Myobatrachidae	+	+			+			
Hylidae	+	+	+	+	+	+	+	+
Leptodactylidae	+			+				
Leiuperidae		+	+		+			
Hyperoliidae	+			+	+			
Dicroglossidae	+							
Ranidae	+	+	+		+			

Note that the Phyllomedusinae belong to Hylidae, and peptides that exclusively can be found in this subfamily are marked with an asterisk. AMPs: antimicrobial peptides; BLP: bombesin-like peptides; BRP: bradykinin-related peptides; Cae: caeruleins; Tachy: tachykinins; Tryp: tryptophyllins; Op: opioids; Sau: sauvagines.

combination of all granular gland products. Thus, no study has focused exclusively on the extract of the tibial gland and it is not always clear whether or not the tibial macrogland has actually been included in the samples analyzed to date. A defensive function for the secretion, however, seems reasonable, especially in light of other studies showing that starving rats will feed on all parts of a freshly killed frog (*Limnodynastes dumerilii*; Myobatrachidae) apart for the tibial gland, which they refuse to eat [20].

Based on the targeted collection and analysis of the secretion from the tibial gland of *P. bicolor*, we report here on the apparent regional localization of skin peptides in this species. Although the skin secretion of *P. bicolor* is otherwise well-studied, and thus supplies sufficient data for comparison, our analyses still identified a phylloseptin-encoding precursor that is new to this species, but which has a 100% identical counterpart in another member of the genus. Moreover, the large-scale surveys of Erspamer, which are based on bioassays, indicated the presence of a BRP in the skin secretion of *P. bicolor* [27], which we confirm in our study to be phyllokinin based on a full-length phyllokininogen sequence. Our findings impact on the questions as to whether skin peptides are expressed identically across an individual animal and also to what extent these peptides are found across closely related species.

2. Materials and methods

2.1. Acquisition of frog skin secretion

One adult individual (female) of *P. bicolor* on loan from City Reptiles (Belfast, UK) was used for this study. The frog was kept in a terrarium and fed with vitamin-dusted crickets three times a week.

Secretion was obtained exclusively from the tibial gland by squeezing it gently with the thumb. This method both ensures that only secretion from this macrogland was obtained and also avoids the known problems with obtaining sufficient secretion in some *Phyllomedusa* species via transdermal electrical stimulation (TES) of the dorsal skin [15,32,49], which has otherwise recently become a favored technique for this purpose, but was found to be ineffective in phyllomedusine frogs [6,10,17]. The slightly transparent white secretion that was discharged (see Fig. 1) was immediately transferred into a sterile polypropylene tube without using any solvents by directly rubbing each hind leg on the inside of the tube. The sample was stored at −20 °C for six months prior to mRNA isolation and cDNA synthesis as established in our lab [12], but without preliminary lyophilization.

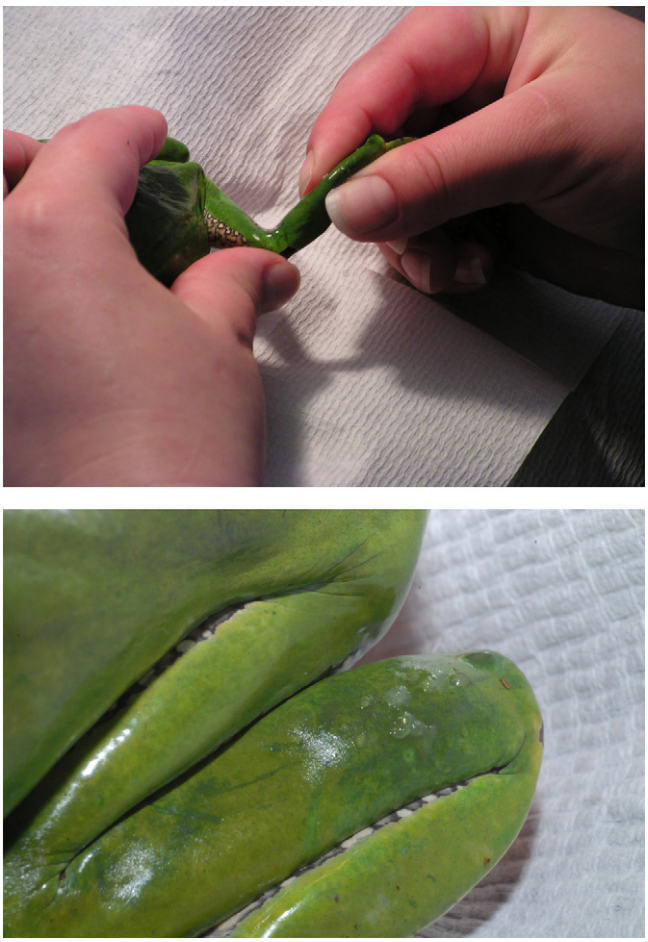

Fig. 1. Optimized collection of tibial gland secretion from *Phyllomedusa bicolor* was achieved by gentle squeezing of these macroglands.

2.2. Construction of a cDNA library from crude skin secretion and subsequent cloning

The entire crude and untreated tibial gland secretion (approx. 5 mg) was placed into 1 mL of cell lysis/mRNA stabilization buffer for 20 min at room temperature. Polyadenylated mRNA was isolated from the stabilization buffer by using magnetic oligo-dT beads (Dynal Biotech, UK) as per the manufacturer's instructions. Isolated mRNA was then reverse-transcribed using a SMART-RACE kit (Clonetech, UK) and resultant cDNA was subjected to 3′-RACE PCR using a NUP primer (supplied with the SMART RACE kit) and a sense primer (S1: 5′-GGCTTYCCTGAAGAAATCTC-3′) that was degenerated at only one position and designed according to an N-terminal sequence -AS/FLKKS- of the highly conserved signal peptide of neobatrachian frog skin AMP precursors [36,50].

The PCR was performed as follows: initial denaturation step: 60 s at 94 °C; 35 cycles: denaturation 30 s at 94 °C, primer annealing for 30 s at 56 °C, extension for 180 s at 72 °C. The resulting PCR fragments (400 bp) were purified (PCR Purification Kit, Jena Bioscience, Germany) cloned using a pGEM-T vector system (Promega Corporation) and sequenced at StarSEQ (Mainz, Germany). All obtained sequences were subjected to online BLAST searches against GenBank for identification and to check for possible contamination.

3. Results

Four different cDNAs that encoded peptide precursors were consistently and repeatedly cloned from the tibial gland secretion

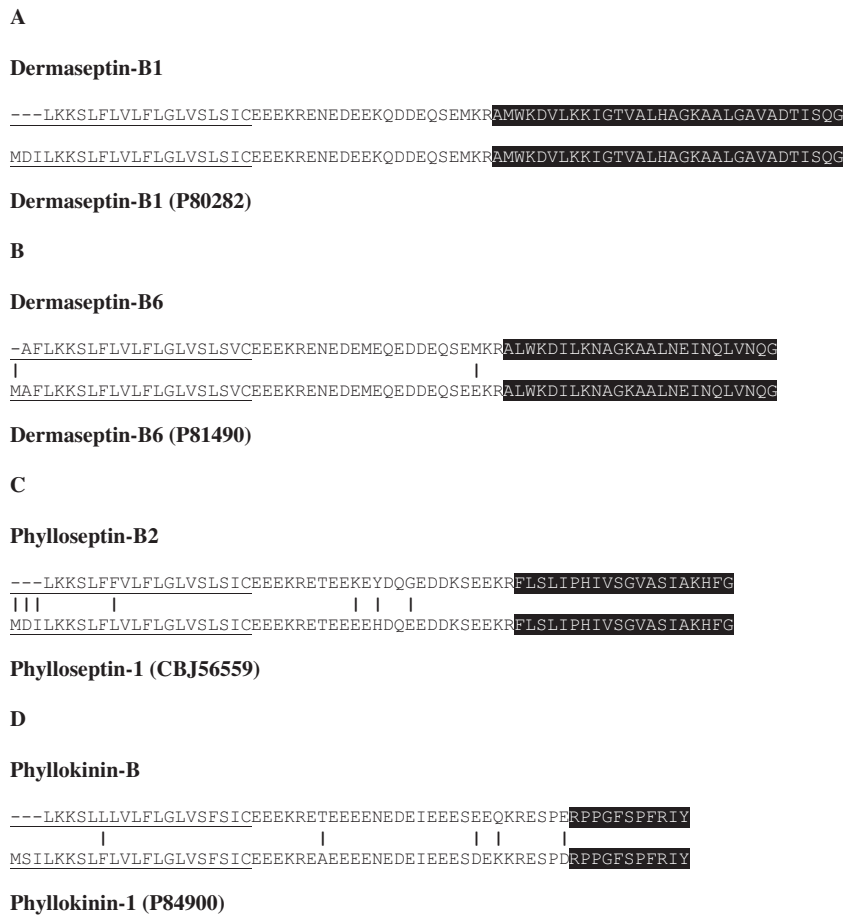

Fig. 2. Four peptide precursors isolated from the tibial gland secretion of *P. bicolor*. Each prepropeptide sequence from our study (upper line) is presented with its putative ortholog from GenBank (lower line; accession number in parentheses). The peptide domains for dermaseptin-B1 (A), dermaseptin-B6 (B), phylloseptin-B2 (C), and phyllokinin (D) are marked in black. Substitutions are marked with vertical bars.

cDNA library of *P. bicolor* (Fig. 2). The inferred amino acid sequences revealed the presence of two previously reported archetypal *P. bicolor* dermaseptins, namely dermaseptin-B1 and dermaseptin-B6 [1,8]. This result was confirmed by BLAST comparisons against GenBank, which revealed 100% sequence identity at the level of the mature peptide as well as virtual identity for the entire precursor structure, with only one substitution present in the spacer region of the two dermaseptins.

The mature peptides of the two remaining skin peptide precursor-encoding cDNAs were 100% identical with the skin peptides phylloseptin-1 from *Phyllomedusa sauvagii* [52] and phyllokinin from *Phyllomedusa hypochondrialis* [11] (Fig. 2). Because these peptide precursors have not been identified previously in *P. bicolor* and their orthology with the related sequences from *P. sauvagii* and *P. hypochondrialis* is not assured, we have designated the encoded mature peptides as phylloseptin-B2 and phyllokinin-B in line with proposed nomenclatural rules [2]. The latter precursor represents the first fully sequenced skin kininogen from *P. bicolor*. Sequence identity between the two precursors identified here and those present in GenBank were slightly lower than for the mature peptide, but still very high. For instance, similarity for both nucleotide and predicted/deduced amino acid sequences was 94% and 89%, respectively, between the prepropeptide of phylloseptin-B2 and that of phylloseptin-1 from *P. sauvagii* and 92% and 92%, respectively, between phyllokinin-B and phyllokininogen from *P. hypochondrialis* (Fig. 3). Across the four *Phyllomedusa* species for which homologous phyllokininogens are known, nucleotide substitutions tend to be neutral at the amino-acid level, either being synonymous (17) or non-synonymous but chemically neutral (8). Only six non-synonymous and not chemically neutral substitutions are present, only two of which (out of seven substitutions in total) appear in the mature peptide domain (Fig. 3).

All sequences from this study have been submitted to GenBank with the following accession numbers: JX294470 for phylloseptin-B2, JX294471 for dermaseptin-B1, JX294472 for dermaspetin-B6, and JX294473 for phyllokinin-B.

4. Discussion

The chemical arsenal of anuran amphibians against predators includes alkaloids, quinones, steroids, amines and peptides [16,22,26]. Aiding this process is the fact that anuran cutaneous glands are apparently predisposed to undergo (sub) specialization in the form of serous gland polymorphism [23], thereby facilitating this highly adapted defensive functioning. Leaf frogs of the genus *Phyllomedusa* present a good example for such skin gland specialization within amphibians, driven in part because of a diurnal lifestyle that includes sunbathing [31]. This behavior requires not only effective anti-predator adaptations, but also protection against desiccation, which is realized by an extra-epidermal lipid layer secreted by a unique third type of skin gland, the lipid glands [4,23,37].

In addition to the use of torpor combined with camouflage, anti-predator adaptations in phyllomedusine frogs include storing large amounts of host-defense peptides within clusters of serous secretory units collectively known as macroglands [48].

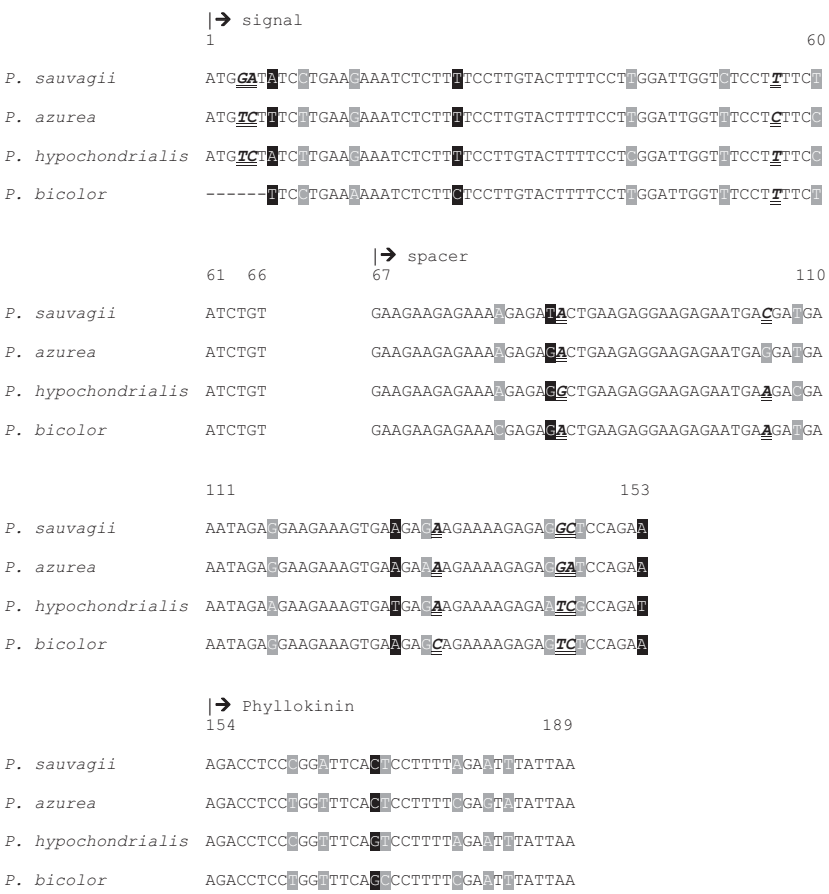

Fig. 3. Alignment of nucleotide sequences of phyllomedusine skin phyllokinins. Sequence data for additional *Phyllomedusa* species derives from GenBank: *P. sauvagii* (AJ549500), *P. azurea* (AM283482), and *P. hypochondrialis* (AM229017). Substitutions with respect to the upper sequence are indicated in gray (synonymous), black (non-synonymous) or double-underlined in bold-italics (non-synonymous, but chemically neutral).

These glands include the anteriorly located parotoid glands (also common in bufonid toads and salamanders) and those associated posteriorly with the hindlimbs such as the paracnemid or tibial glands. Thus, in combination with their particular wiping behavior for the distribution of the secretory lipids, phyllomedusine frogs can simultaneously cover their body with these defensive peptides given that the frogs repeatedly touch macroglandular regions such as the tibial and parotoid glands during wiping [4,31].

This behavior, in combination with the sampling procedures commonly used for skin peptides, however, might disguise the presence of important variation in glandular composition within an individual. Indeed, separate sampling of the skin and parotoid secretions from bufonid toads [39] support precisely this scenario in these animals. Despite being generally well sampled, the secretions of the macroglands in *Phyllomedusa* have not been examined in isolation. At best, the tibial glands of a related species, *P. sauvagii*, have been specifically sampled [10,13,14]; however, these secretions were subsequently combined with those of the parotoid glands for molecular and biochemical characterization.

Our cloning study of the constructed cDNA library for the tibial gland of *P. bicolor* revealed the precursor-encoding cDNAs for two AMPs, dermaseptin-B1 and dermaseptin-B6, reported for this species [1,8] as well as for phyllokinin. Although the latter has long been known to be present in the skin secretion of *P. bicolor* for some time [27], its precursor-encoding cDNA has not been reported until now. In the present study we successfully cloned the phyllokinin precursor, which represents the first skin kininogen from this species. More importantly, despite the fact that our cloning study was not systematic, we were still able to identify a novel peptide for *P. bicolor*, namely phylloseptin-B2.

Intriguingly, both phyllokinin and phylloseptin-B2 show high sequence similarity at the amino-acid level to counterparts in other species. For phylloseptin-B2, a classical AMP that eradicates entire biofilms [52], this result was unexpected given that two identical AMPs are not expected to be found in two (or more) different species [24]. Because AMPs function by disrupting cell membranes through their cationic, amphipathic and α-helical nature [40], their primary structures can often adopt mutations without losing efficacy and even undergo strong positive selection [47] so long as the crucial chemical features for antimicrobial activity are maintained. However, increased sampling in recent years has revealed that although cross-species sharing of AMPs remains the exception rather than the rule, it is more frequent than previously believed, with examples from numerous frog families, including ascaphins [18], magainins [35], bombinin-like peptides (compare [38,42]), caerin 1.1 [33] and nigrocin-2 (see GenBank CAM57304, CAM35483 and [44,53]), the latter being as yet the broadest case for AMP sharing (across three genera) known within anuran amphibians.

Our study provides another example of this phenomenon, with a phylogenetic perspective hinting that phylloseptin might even be relatively broadly shared within *Phyllomedusa* given that *P. sauvagii* and *P. bicolor* are probably not sister species [30]. Although divergence time information within Phyllomedusinae is lacking, it remains that *P. sauvagii* and *P. bicolor* are separated by numerous other species (e.g., *Phyllomedusa camba* + the *Phyllomedusa tarsius* group, and *Phyllomedusa boliviana*). Thus, it remains that these and

possibly additional species within Phyllomedusinae also have identical phylloseptin homologs.

By contrast, the high sequence similarity among phyllomedusine phyllokinins (Fig. 3) does derive from the mode of action of this peptide. Phyllokinin is strongly vasoactive and belongs to the family of bradykinin-related peptides (BRPs) that target endogenous bradykinin receptors of the ingesting predator. To assure these biological effects, the primary structures of such ligand-mimicking peptides tend to be highly conserved by selective pressure. In addition, the similarity of the phyllokinins observed here must mirror that of the natural spectrum of predators of the frog species (e.g., snakes, birds, mammals, or fish) because of the ligand binding specificity of the receptors. Although some sequence divergence among phyllomedusine phyllokinins and phyllokininogens is present, only six of the 31 total substitutions result in a different amino acid with chemically different properties, of which only two of those occur in the mature peptide (Fig. 3). Again a fair degree of conservation for phyllokinin is suggested.

An interesting future course of research would be an evolutionary comparison of BRPs across a broader taxonomic sample. As proteomic studies have shown, BRPs are also produced in the skin secretion of other phyllomedusine frogs (*Phyllomedusa nordestina*, *Phyllomedusa rohdei*, *Agalychnis (formerly Pachymedusa) dacnicolor* and *Phasmahyla jandaia*) [3,17,41,45] and kininogens are also known from more distantly related taxa (e.g., Bombinatoridae, Ranidae) [9,46]. However, as for AMPs (see [36]), we hypothesize that the latter might have evolved convergently due to their distinct structural architecture. In comparing members of this gene family, however, it must be remembered that all types of bradykinin modifications (proline hydroxylation, tyrosine sulfation) apparently derive from a single precursor by differential post-translational processing [10] making isolation of the entire precursor unit essential. This finding also opens the possibility that bradykinin modifications might also occur in different glands of *P. bicolor*, leading perhaps to even greater regional specialization of skin peptides than is currently documented. Nevertheless, our preliminary study, which focused only on the transcriptome of the tibial gland, demonstrates that targeted sampling of different granular glands presents good prospects to discover novel peptide-encoding cDNAs in amphibians, even in well-studied species. Furthermore, we would argue on the basis of our results that future studies should not combine secretions from different glands, particularly those contents from specialized macroglands, because doing so could mask the extent of variation within the same individual, which might otherwise lend important insights into the evolution and ecology of frog skin chemistry.

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