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Peptide defenses of the Cascades frog *Rana cascadae*: implications for the evolutionary history of frogs of the *Amerana* species group

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ABSTRACT

The Cascades frog *Rana cascadae* belongs to the *Amerana* (or *Rana boylii*) group that includes six additional species from western North America (*R. aurora*, *R. boylii*, *R. draytonii*, *R. luteiventris*, *R. muscosa*, and *R. pretiosa*). *R. cascadae* is particularly susceptible to pathogenic microorganisms in the environment and populations have declined precipitously in parts of its range so that the protection afforded by dermal antimicrobial peptides may be crucial to survival of the species. Peptidomic analysis of norepinephrine-stimulated skin secretions led to the identification of six peptides with differential cytolytic activities that were present in high abundance. Structural characterization showed that they belonged to the ranatuerin-2 (one peptide), brevinin-1 (one peptide), and temporin (four peptides) families. Ranatuerin-2CSa (GILSSFKGVAKGVAKDLAGKLLETLKCKITGC) and brevinin-1CSa (FLPILAGLAAKIVPKLFCLATKKC) showed broad spectrum antibacterial activity (MIC $\leq$ 32 μM against *Escherichia coli* and *Staphylococcus aureus*) but only brevinin-1CSa was strongly hemolytic against human erythrocytes (LC_{50} = 5 μM). The taxonomy of ranid frogs is currently in a considerable state of flux. The ranatuerin-2 gene is expressed in all members of the *Amerana* group studied to-date and cladistic analysis based upon a comparison of the amino acid sequences of this peptide indicates that *R. cascadae*, *R. muscosa* and *R. aurora* form a clade that is distinct from one containing *R. draytonii*, *R. boylii*, and *R. luteiventris*. This conclusion is consistent with previous analyses based upon comparisons of the nucleotide sequences of mitochondrial genes.

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1. Introduction

Frogs belonging to the genus *Rana*, often referred to as ''true frogs'', are a relatively successful group of anurans with more than 250 species distributed worldwide, except for the Polar Regions, southern South America and most of Australia. Approximately one-quarter of known species are found in the New World [12]. On the basis of morphological criteria, five species (*R. aurora*, *R. boylii*, *R. cascadae*, *R. muscosa*, and *R. pretiosa*) that occur in the western states of the U.S.A. were originally classified together in the *Amerana* species group (also known as the *R. boylii* group) [10]. Subsequently, *R. pretiosa* was divided into two species with distinct geographical ranges (*R. pretiosa* in the west and *R. luteiventris* in the east) [18]. More recently, the red-legged frogs, traditionally regarded as a single polytypic species with two subspecies, the northern red-legged frog *R. aurora aurora* and the California red-legged frog *R. aurora draytonii* have also been classified as two separate species, *R. aurora* and *R. draytonii* [33]. Molecular data based upon mitochondrial DNA sequences [21,24] support earlier claims [35] proposing monophyletic status for the *Amerana* and suggest that the group is approximately 8 million years old. However, phylogenetic and evolutionary relationships among members of the group have yet to be fully resolved.

The Cascades frog *R. cascadae* is a medium sized (adult males 5–6 cm, adult females 6–7.5 cm), diurnal frog that inhabits mountainous streams and ponds in forested areas in the Cascade Mountains in a band from Washington to northern California [29]. The species is still fairly abundant in parts of its range but has disappeared completely from other areas, particularly in the southern edge of its range in California [9,13,36]. Multiple factors have been proposed for this decline and include drought [13], destruction of habitat due to logging and agriculture [9,13], introduction of aquatic predators such as trout and the bullfrog *R. catesbeiana* [36], wind-borne agrochemicals [8,9], and sensitivity of oocytes to increased UV-B radiation [22,23]. *R. cascadae* is particularly susceptible to the pathogenic oomycete *Saprolegnia ferax* [22,23] and to the chytrid fungus *Batrachochytrium dendrobatidis* [15]. Consequently, the ability of the animal to mount a defense against these and other microorganisms in the environment may be of critical importance to the survival of the species [27].

Cationic α-helical peptides with broad-spectrum antibacterial and antifungal activities are synthesized in the skins of the majority, but by no means all, species of ranid frogs and represent a component of the animal's system of innate immunity [5,19]. The peptides may be grouped into families on the basis of limited structural similarity but the variation in amino acid sequences of homologous peptides is considerable and it is rare that orthologs have an identical primary structure, even when they are from species that are quite closely related phylogenetically [5]. Previous studies have led to the purification and structural characterization of antimicrobial peptides from the skin secretions of several species belonging to the *Amerana* group: *R. luteiventris* [17], *R. boylii* [7], *R. aurora* [6], *R. draytonii* [3], and *R. muscosa* [32]. The present investigation extends this program by describing the purification and characterization of multiple peptides with antimicrobial activity from skin secretions of *R. cascadae*. The nomenclature used to describe the peptides is the same as that used for other *Rana* skin peptides with CS indicating the species and the isoforms designated by lower case letters.

2. Materials and methods

2.1. Collection of skin secretions

Animals were collected during July in Gumboot Creek, Shasta Trinity National Forest, Siskiyou County, California, under permit (California Department of Fish and Game scientific collection permit 801031-02). Skin secretions were obtained by a procedure previously described [3,32]. In brief, adult and subadult specimens of indeterminate sex ($n = 8$; weight 5–15 g) were injected bilaterally with norepinephrine (10 nmol/g body wt.) and individually placed in distilled water (100 ml) for 15 min. The combined secretions and washings were acidified by addition of trifluoroacetic acid (0.5 ml) and immediately frozen. After stimulation, all animals were released unharmed at the exact sites of collection.

2.2. Antimicrobial and hemolytic assays

Purification of the peptides was monitored by incubating lyophilized aliquots of chromatographic effluent in Mueller–Hinton broth (50 μl) with an inoculum (50 μl of 10^6 colony forming units ml^{-1}) from a log-phase culture of reference strains *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25726) in 96-well microtiter cell-culture plates for 18 h at 37 °C in a humidified atmosphere of air. After incubation, the absorbance at 630 nm of each well was determined using a microtiter plate reader. In order to monitor the validity and reproducibility of the assays, incubations were carried out in parallel with increasing concentrations of ampicillin. Minimum inhibitory concentration (MIC) was measured by a standard microdilution method [26] and was taken as the lowest concentration of peptide where no visible growth was observed.

Peptides in the concentration range 1–200 μM were incubated with washed human erythrocytes (2×10^7 cells) from a healthy donor in Dulbecco's phosphate-buffered saline, pH 7.4 (100 μl) for 1 h at 37 °C. After centrifugation (12,000 × *g* for 15 s), the absorbance at 450 nm of the supernatant was measured. A parallel incubation in the presence of 1% (v/v) Tween-20 was carried out to determine the absorbance associated with 100% hemolysis. The LC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis in three independent experiments.

2.3. Peptide purification

Skin secretions were passed at a flow rate of 2 ml min^{-1} through 8 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70.0:29.9:0.1, v/v/v) and freeze-dried. The lyophilisate was redissolved in 0.1% (v/v) trifluoroacetic acid/water (4 ml) and injected onto a (2.2-cm × 25-cm) Vydac 218TP1022 (C-18) reverse-phase HPLC column (Separations Group, Hesperia, CA) equilibrated with

0.1% (v/v) trifluoroacetic acid/water at a flow rate of 6.0 ml min^{-1}. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 63% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 nm and 280 nm and fractions (1 min) were collected. The abilities of freeze-dried aliquots (50 μl) of the fractions to inhibit the growth of *S. aureus* and *E. coli* were determined as described in the previous section. Fractions with antimicrobial activity were successively chromatographed on a (1 × 25-cm) Vydac 214TP510 (C-4) column and a (1 × 25-cm) Vydac 219TP510 (phenyl) column. The concentration of acetonitrile in the eluting solvent was raised from 21% to 56% over 50 min and the flow rate was 2.0 ml min^{-1}.

2.4. Structural characterization

The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 494 Procise sequenator. MALDI-TOF mass spectrometry was carried out using a Bruker Ultraflex Tof/Tof instrument (Bruker Daltonics Inc., Billerica, MA) using α-cyano-4-hydroxycinnamic acid as matrix. Spectra were acquired in reflector mode and were internally calibrated using a standard peptide mix. The resulting accuracy is better than 0.05%. Amino acid composition analyses were performed by the University of Nebraska Medical Center Protein Structure Core Facility (Omaha, NE).

2.5. Cladistic analysis

Phylogenetic analysis of the ranatuerin-2 sequences was performed using the Phylogeny Inference Program package, PHYLIP, version 3,57c (Felsenstein, J., University of Washington, Seattle WA, 1993). The stability of the tree was tested by bootstrap resampling analysis of 100 replicates computed with the SEQBOOT program. Genetic distances between each pair of amino acid sequences were calculated using the PROTDIST program based on the categories model according to chemical categorization of amino acids. From this distance matrix, the phylogenetic tree was generated by the neighbor-joining method of the NEIGHBOR program and displayed by DRAWGRAM. The amino acid sequence of ranatuerin-2SPa from *Rana septentrionalis* [1] was used as outgroup to polarize the ingroup taxa.

3. Results

3.1. Purification of the peptides

The skin secretions from *R. cascadae*, after concentration and partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 preparative reversed-phase HPLC column (Fig. 1). Aliquots of the fractions were tested for their ability to inhibit growth of Gram-negative bacteria, *E. coli* and Gram-positive bacteria, *S. aureus*. Antimicrobial activity was associated with the incompletely resolved peaks designated 1–6. Subsequent structural analysis demonstrated that peak 1 contained ranatuerin-2CSa, peaks 2 and 3 contained a mixture of brevinin-1CSa and temporin-1CSa, peaks 4 and 5 contained a mixture of temporin-1CSb and temporin-1CSc, and peak 6 contained temporin-1CSd. The individual antimicrobial peptides were purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry, by further chromatography on Vydac C-4 and Vydac phenyl columns. The methodology is illustrated by separation of temporin-1CSa and brevinin-1CSa on a Vydac C-4 column (Fig. 2) and final purification of each component on a Vydac phenyl column (Fig. 3B and C). The final yields of purified peptides (μmol) were ranatuerin-2CSa 6.5, brevinin-1CSa 1.1, temporin-1CSa 4.8, temporin-1CSb 5.4, temporin-1CSc 1.5, and temporin-1CSd 1.7.

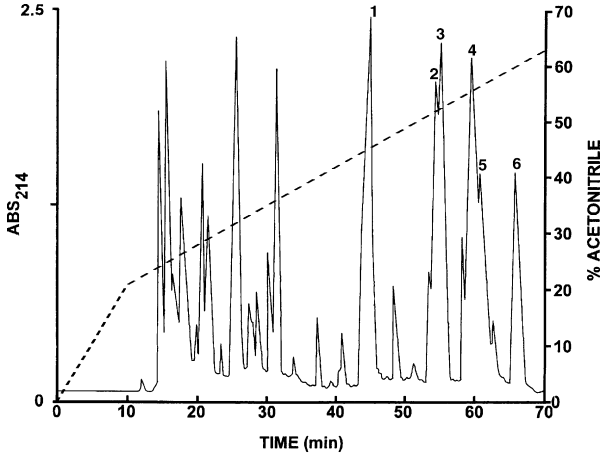

Fig. 1 – Reverse-phase HPLC on a semipreparative Vydac C-18 column of norepinephrine-stimulated skin secretions from *Rana cascadae*. The peaks designated 1–6 were associated with antimicrobial activity and were subjected to further purification. The dashed line shows the concentration of acetonitrile in the eluting solvent.

3.2. Structural characterization

The primary structures of the antimicrobial peptides isolated from the skin secretions were established by automated

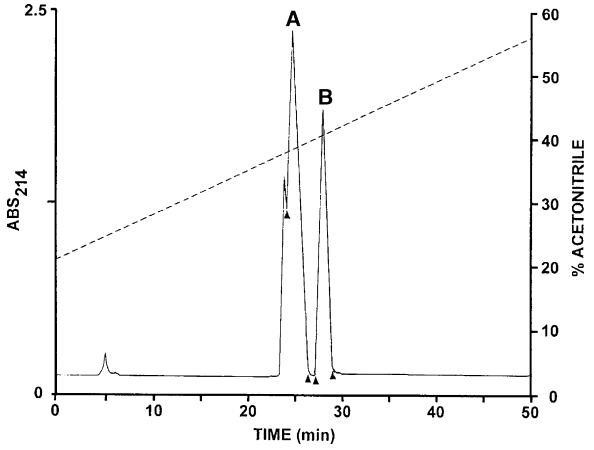

Fig. 2 – Separation of temporin-1CSa (peak A) and brevinin-1CSa (peak B) on a Vydac C-4 column. The arrowheads show where peak collection began and ended.

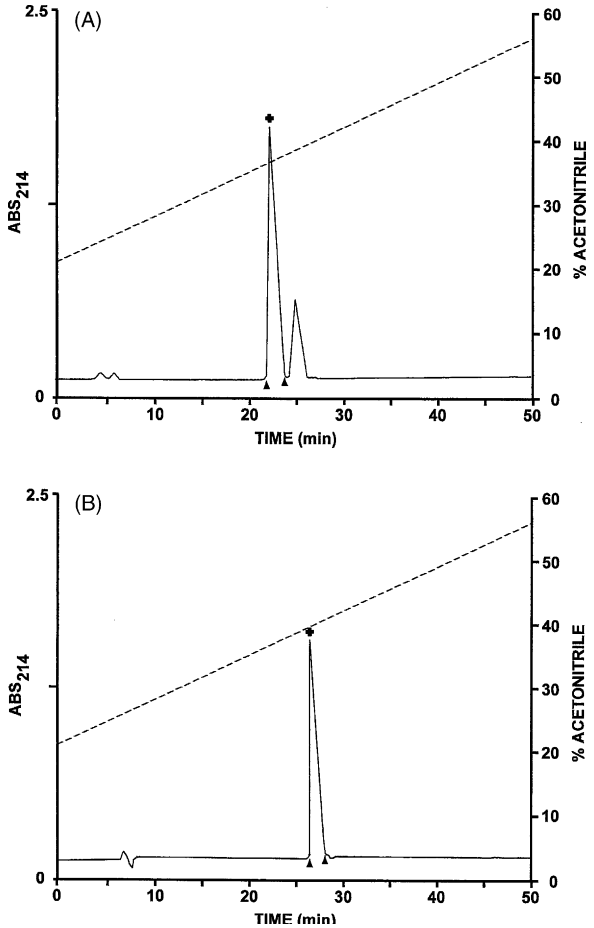

Fig. 3 – Purification to near homogeneity on a Vydac phenyl column of (A) temporin-1CSa, and (B) brevinin-1CSa. (+) denotes the peak associated with antimicrobial activity.

Edman degradation and their amino acid sequences are shown in Fig. 3. As there was some ambiguity with regard to identification of the C-terminal amino acid residue in the temporin peptides, the sequences were confirmed by amino acid composition analysis [Found: *temporin-1CSa* Ser 1.1 (1), Pro 1.1 (1), Gly 2.2 (2), Val 0.7 (1), Ile 0.7 (1), Leu 4.7 (5), Phe 1.1 (1), Lys 1.0 (1); *temporin-1CSb* Ser 0.8 (1), Pro 1.0 (1), Gly 1.8 (2), Ile 1.5 (2), Leu 4.7 (5), Phe 1.0 (1), Lys 1.0 (1);. *temporin-1CSc* Thr 1.0 (1), Ser 1.1 (1), Pro 1.1 (1), Gly 2.1 (2), Val 1.0 (1), Leu 5.6 (6), Phe 1.1 (1); *temporin-1CSd* Asx 2.0 (2), Thr 1.1 (1), Gly 1.1 (1), Ala 1.1 (1), Val 1.0 (1), Ile 0.9 (1), Leu 3.7 (4), Phe 1.1 (1), Lys 1.8 (2) residues/mol peptide]. Figures in parentheses show the number of residues predicted from the proposed sequences. MALDI-TOF mass spectrometry demonstrated the presence of a C-terminally α-amidated residue at the C-terminus of each of the temporin peptides and the presence of a disulfide bridge in the ranatuerin-2 and brevinin-1 peptides (Fig. 4).

Table 1 – Minimum inhibitory concentrations (μM) and hemolytic activities against human erythrocytes (μM) of peptides isolated from skin secretions of *Rana cascadae*

Peptide	*E. coli*	*S. aureus*	LD_{50}
Ranatuerin-2CSa	4	8	150
Brevinin-1CSa	32	2	5
Temporin-1Csa	128	8	75
Temporin-1CSb	128	8	95
Temporin-1CSc	>128	64	>300
Temporin-1CSd	64	16	50

3.3. *Antimicrobial and hemolytic activities*

The abilities of the purified peptides from *R. cascadae* to inhibit the growth of *S. aureus* and *E. coli* and to lyse human erythrocytes are quantified in Table 1.

4. Discussion

The present investigation supports the conclusion of a previous analysis of the *Aquarana* group of North American frogs [4] that the distribution and amino acid sequences of antimicrobial peptides in skin secretions may be used to complement other molecular analyses, such as comparison of nucleotide sequence of mitochondrial genes, in elucidating phylogenetic and evolutionary relationships between species of ranid frogs. The taxonomy of ranid species is currently in a considerable state of flux. The application of molecular techniques of phylogenetic analysis has led to a reappraisal of taxonomic classifications that had been established using morphological criteria and behavioral characters. A comprehensive analysis of New World ranids based upon comparisons of nucleotide

		M_r (obs)	M_r (calc)
Ranatuerin-2CSa	GILSSFKGVAKGVAKDLAGKLLETLKCKITGC	3245.9	3245.8
Brevinin-1CSa	FLPILAGLAAKIVPKLFCLATKKC	2555.8	2555.5
Temporin-1CSa	FLPIVGKLLSGLL.NH_2	1368.1	1367.9
Temporin-1CSb	FLPIIGKLLSGLL.NH_2	1382.0	1381.9
Temporin-1CSc	FLPLVTGLLSGLL.NH_2	1362.8*	1340.9
Temporin-1CSd	NFLGTLVNLAKKIL.NH_2	1542.0	1542.0

*Na adduct

Fig. 4 – Amino acid sequences, observed molecular masses (M_r obs), and calculated molecular masses (M_r calc) of the antimicrobial peptides isolated from skin secretions of *R. cascadae*.

Table 2 – Distribution of the molecular forms of the antimicrobial peptides isolated from the skins of frogs belonging to the *Amerana* species group

	Ranatuerin-2	Brevinin-1	Temporin
R. draytonii	2	4	3
R. aurora	1	2	1
R. boylii	2	3	1
R. cascadae	1	1	4
R. muscosa	2	0	1
R. luteiventris	2	2	3

The values in the table show the number of peptides belonging to a particular family that are produced by that species.

sequences from the mitochondrial genome has been carried out [21] that supports monophyletic status for the *Amerana* and suggests that the group is more closely related to the *R. temporaria* group of Eurasian ranids than to the remaining species of North American ranids that are classified together in the *Novirana*. This taxonomic organization has, however, been criticized [11]. It has been proposed more recently that the North American ranids should be divided into two groups with the genus *Rana* reserved for the *Amerana* and all other ranid united in the monophyletic genus *Lithobates* [14] but this classification has also been subject to criticism [20].

The data in the present study support the claim that *Amerana* constitutes a monophyletic group. All members of the group studied to-date, including *R. cascadae* (*R. pretiosa* has not yet been investigated) produce only antimicrobial peptides that belong to the ranatuerin-2 [16], brevinin-1 [25], and temporin [34] families. The number of peptides belonging to each family is different for each species and the distribution is shown in Table 2. The amino acid sequences of orthologous gene products are compared in Fig. 5. Ranatuerin-2 has been found in skin secretions of all members of the *Amerana* (Table 2), as well as in a wide range of other North American [5] and Eurasian [30] ranid species. Cladistic analysis based upon the amino acid sequence of ranatuerin-2 demonstrates that the peptides segregate into two major clades comprising (a) *R. cascadae*, *R. muscosa*, and *R. aurora* and (b) *R. draytonii*. *R. boylii* and *R. luteiventris* (Fig. 6). This analysis suggests a sister-group relationship between *R. cascadae* and *R. muscosa* and between *R. draytonii* and *R. boylii* and supports the conclusion of an earlier study that *R. aurora* and *R. draytonii* are not subspecies of a single polytypic species [3]. While only a single member of the family was identified in the skins of *R. cascadae* and *R. aurora*, putative gene duplications within the *R. luteiventris*, *R. draytonii*, and *R. muscosa* lineages have led to the expression of paralogous genes. A comparison of the amino acid sequences of the ranatuerin-2 peptides (Fig. 5) suggests that the duplicated genes have undergone an accelerated rate of mutation as ranatuerin-2AUb (from *R. aurora*) and ranatuerin-2BYb (from *R. boylii*) both contain a four residue deletion and in ranatuerin-2DRb (from *R. draytonii*), the strongly conserved Val[9] residue is replaced by Ile and the Ile[29] residue is replaced by Met.

Our conclusion based upon the amino acid sequences of dermal antimicrobial peptides are fully consistent with analyses based upon the nucleotide sequences of mitochondrial genes. Both Macey et al. [24] and Hillis and Wilcox [21] found strong support for a clade comprising *R. cascadae*, *R. aurora*, and *R. muscosa* with the former study indicating a sister-group relationship between *R. cascadae* and *R. muscosa*, as in this investigation, and the latter study indicating a sister-group relationship between *R. cascadae* and *R. aurora*. Weaker support for an association between *R. boylii* and *R. luteiventris* was obtained. A limitation of both these studies is that neither differentiated between *R. aurora* and *R. draytonii*.

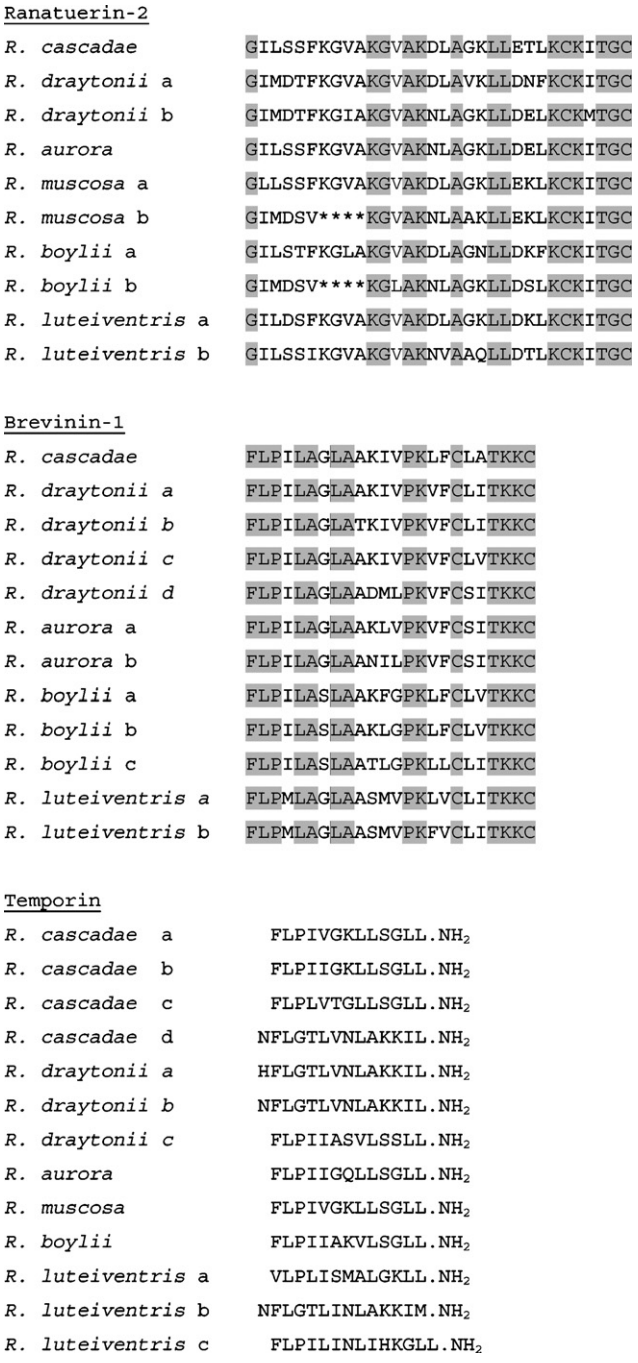

Fig. 5 – A comparison of the primary structures of peptides belonging to the ranatuerin-2, brevinin-1, and temporin families isolated from frogs belonging to the *Amerana* group. The shaded residues are conserved. In order to maximize structural similarity, residue deletions denoted by (*) have been introduced in some sequences.

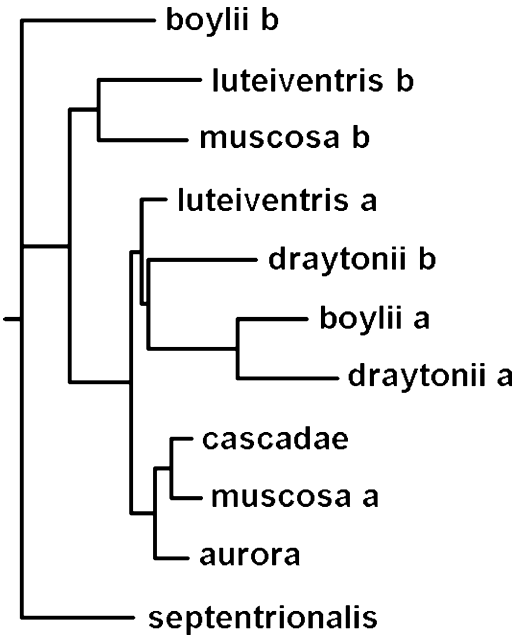

Fig. 6 – A phylogenetic tree based upon the amino acid sequences of ranatuerin-2 peptides isolated from the skins of frogs belonging to the *Amerana* species group. Ranatuerin-2SPa from *R. septentrionalis* was used as outgroup.

A noteworthy feature of the present work was the extremely high concentration of antimicrobial peptides present in norepinephrine-stimulated skin secretions. The quantity of purified peptides obtained from eight specimens was approximately 57 mg. These components were to varying degrees active against both Gram-negative and Gram-positive bacteria. Although injection of norepinephrine does not constitute a physiological stimulus, the data suggest the *R. cascadae* is capable of mounting a vigorous defense against bacterial invasion. Further studies are required to determine whether the peptides isolated from skin secretions inhibit the growth of pathogens present in the frog's environment, such as the chytrid fungus, *B. dendrobates* that has been responsible for amphibian declines worldwide [31].

The emergence in all regions of the world of strains of pathogenic bacteria and fungi with resistance to commonly used antibiotics constitutes a potentially serious threat to public health and has necessitated a search for novel types of antimicrobial agent to which the microorganisms have not been exposed [28]. Anti-infectives based on peptides synthesized in frog skin have been considered as potential therapeutic agents [2]. As shown in Table 1, ranatuerin-2, displaying broad spectrum antibacterial activity but low hemolytic activity, represents such a candidate for drug development. Similarly, temporin-1CSc, which lacks the basic amino acid residue that is usually present in members of the temporin family, is completely devoid of hemolytic activity but is still capable of inhibiting the growth of the Gram-positive bacteria, *S. aureus*. Brevinin-1CSa and temporin-1CSd show growth-inhibitory activity against Gram-positive and Gram-negative bacteria but their relatively high hemolytic activities limit their potential for clinical use.

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