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Evidence from peptidomic analysis of skin secretions that the red-legged frogs, *Rana aurora draytonii* and *Rana aurora aurora*, are distinct species

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ABSTRACT

The northern red-legged frog *Rana aurora aurora* and the California red-legged frog *Rana aurora draytonii* are traditionally classified together in the same species group. Ten peptides with antimicrobial activity were isolated from norepinephrine-stimulated skin secretions of *R. aurora draytonii* and purified to near homogeneity. The peptides were identified as belonging to the ranatuerin-2 family (two peptides), brevinin-1 family (four peptides), temporin family (three peptides), and a novel peptide, RV-23 (RIGVLLARLPKLFSLFKLMGKKV) that has limited structural similarity to the bee venom peptide, melittin. This distribution of peptides contrasts with that found previously in skin secretions from *R. aurora aurora* collected under the same conditions and at the same time of year (one ranatuerin-2 peptide, two brevinin-1 peptides, and one temporin peptide). The variation in amino acid sequences between corresponding *R. aurora draytonii* and *R. aurora aurora* peptides is comparable with the variation in sequences of orthologs from other members of the *Amerana* group of New World ranid frogs (*Rana boylii*, *Rana muscosa*, and *Rana luteiventris*). It is proposed, therefore, that the red-legged frogs should be regarded as separate species (*R. aurora* and *R. draytonii*) within the *Amerana* group rather than conspecific subspecies. The data emphasize that amino acid sequences of antimicrobial peptides in skin secretions may be used to infer taxonomic and phylogenetic relationships between species of ranid frogs.

1. Introduction

Frogs belonging to the genus *Rana* (Neobatrachia, Ranidae) often referred to as "true frogs," represent a diverse group comprising more than 250 species that are distributed worldwide, except for the Polar Regions, southern South America, and most of Australia. Approximately one-quarter of the ranid species are to be found in the New World, with greatest species diversity in the southern United States and Mexico [20]. Morphological differences between species are often slight so

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that taxonomic classification of specimens can be difficult, and unambiguous identification of individuals is especially challenging in regions where several species coexist and produce hybrids [6]. Similarly, the fossil record of the ranids is poor and therefore evolutionary relationships within the genus are incompletely understood. In recent years, molecular techniques of phylogenetic analysis, particularly the comparison of nucleotide sequences of orthologous genes, have proved to be valuable in complementing ''classical'' techniques based on morphological analyses for elucidating the evolutionary history of the ranid frogs [1,19,20,22].

Peptides with broad-spectrum antibacterial and antifungal activities are synthesized in the skins of several, but by no means all, species of anurans (frogs and toads) and represent a component of the animal's system of innate immunity that defends the frog against invasion by pathogenic microorganisms [21,27]. Ranid frogs have proved to be a particularly rich source of such peptides [4,6,34]. On the basis of limited structural similarity, these antimicrobial peptides may be grouped together in families that share a common evolutionary origin but the variation in amino acid sequences of homologous peptides is considerable [6,16]. Virtually no single peptide from one species is found with an identical amino acid sequence in another. Consequently, determination of the primary structures of these peptides can be used to facilitate unambiguous identification of species and provide valuable insight into phylogenetic relationships.

On the basis of morphological criteria, six species (*Rana aurora, Rana boylii, Rana cascadae, Rana luteiventris, Rana muscosa,* and *Rana pretiosa*) that occur in the western states of the U.S.A. have been classified together in the *Amerana* species group [13], also known as the *R. boylii* group [22]. Recent molecular data based on mitochondrial DNA sequences support monophyletic status for the *Amerana* and the extent of sequence divergence suggests that the group is approximately 8 million years old [20,22]. However, phylogenetic relationships among members of the group have yet to be fully resolved.

The red-legged frogs *R. aurora* [2] are restricted to the Pacific coastal regions of North America, from southern British Columbia to northern Baja California. Traditionally, *R. aurora* has been 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* [3]. However, the two subspecies show appreciable differences in size and reproductive behavior [17], and molecular analyses of allozymes [18] and comparison of the nucleotide sequences of a fragment of the mitochondrial cytochrome *b* gene [33] have suggested *aurora* and *draytonii* should be classified as separate species with a narrow zone of overlap in northern California.

The red-legged frogs have disappeared from much of their range and declines in the populations of the California red-legged frog have been particularly dramatic. Since 1996, *R. aurora draytonii* has been listed as threatened under the U.S. Endangered Species Act. Wind-borne agrochemicals and habitat destruction due to urbanization have been implicated in population declines [11,12].

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* [16], *R. boylii* [8], *R. aurora aurora* [7], and *R. muscosa* [30]. The present investigation extends this program by describing the characterization of multiple peptides with antimicrobial activity from skin secretions of *R. aurora draytonii*. The nomenclature used to describe the peptides is the same as that used for other *Rana* skin peptides with DR indicating the species and the isoforms designated by lower case letters. The data strongly support the hypothesis that *R. aurora aurora* and *R. aurora draytonii* should be classified as separate species (*R. aurora* and *R. draytonii*) within the *Amerana* group rather than as conspecific subspecies.

2. Materials and methods

2.1. Collection of skin secretions

Animals were collected during early summer at Morgan Territory Regional Preserve, Alameda County, CA, USA, with permits from the California Department of Fish and Game (#801031-02) and U.S. Fish and Wildlife Service (#TE839092). Skin secretions from *R. aurora draytonii* were collected by a procedure previously described [7]. In brief, three juvenile specimens of indeterminate sex (weights 8.0, 8.2, and 8.6 g) were injected bilaterally with norepinephrine (10 nmol/g body weight) and placed in distilled water (100 ml) for 15 min. The combined secretions and washings were acidified by addition of trifluoroacetic acid (1 ml) and passed at a flow rate of 2 ml min^{-1} through 4 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70.0:29.9:0.1, v/v/v) and freeze-dried. After stimulation, all animals were released unharmed at the exact sites of collection. Collection of skin secretions from *R. aurora aurora* has been described previously [7].

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* (NCTC 8325) and *Escherichia coli* (ATCC 25922) in 96-well microtiter cell-culture plates for 18 h at 37 °C in a humidified atmosphere of air. Incubations with *Candida albicans* (ATCC 90028) were carried out in RPMI 1640 medium for 48 h at 35 °C. 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 with bacteria were carried out in parallel with increasing concentrations of bacitracin and incubations with *C. albicans* in parallel with amphotericin B. 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

The skin secretions, after partial purification on Sep-Pak cartridges, were redissolved in 0.1% (v/v) trifluoroacetic acid/water (2 ml) and injected onto a (1 cm × 25 cm) Vydac 218TP510 (C-18) reverse-phase HPLC column (Separations Group, Hesperia, CA, USA) equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 2.0 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 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 cm × 25 cm) Vydac 214TP510 (C-4) column and a (1 cm × 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}. The purification of peptides from *R. aurora aurora* skin secretions has been described previously [7].

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 Voyager DE-PRO instrument (Applied Biosystems) that was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 20 kV. The instrument was calibrated with peptides of known molecular mass in the 2000–4000 Da range. The accuracy of mass determinations was ±0.02%. Amino acid composition analyses were performed by the University of Nebraska Medical Center Protein Structure Core Facility (Omaha, NE, USA).

2.5. Peptide synthesis

Peptide RV-23, temporin-1DRa, and temporin-1DRb were supplied in crude form by GL Biochem (Shanghai, China) and purified to near homogeneity by reverse-phase HPLC on a (2.2 cm × 25 cm) Vydac 218TP1022 (C-18) column equilibrated with acetonitrile/water/trifluoroacetic acid (21.0:78.9:0.1) at a flow rate of 6 ml min^{-1}. The concentration of acetonitrile was raised to 56% over 60 min using a linear gradient. The final purity of the synthetic peptides was >95% and their identities were confirmed by electrospray mass spectrometry.

3. Results

3.1. Purification of the peptides

The skin secretions from *R. aurora draytonii*, after concentration and partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 semipreparative reverse-phase HPLC column (Fig. 1A). The elution profile under the same condition of chromatography of a sample of norepinephrine-stimulated skin secretions from *R. aurora aurora* is shown in Fig. 1B. Aliquots of the *R. aurora draytonii* 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 well-defined peaks designated 1–9. Subsequent structural analysis demonstrated that peak 1 contained ranatuerin-2DRa, peak 2 contained RV-23, peak 3 contained ranatuerin-2DRb, peak 4 contained temporin-1DRa, peak 5 contained brevinin-1DRa, peak 6 contained a mixture of brevinin-1DRb and brevinin-1DRc, peak 7 contained brevinin-1DRd, peak 8 contained temporin-1DRb, and peak 9 contained temporin-1DRc. Under the

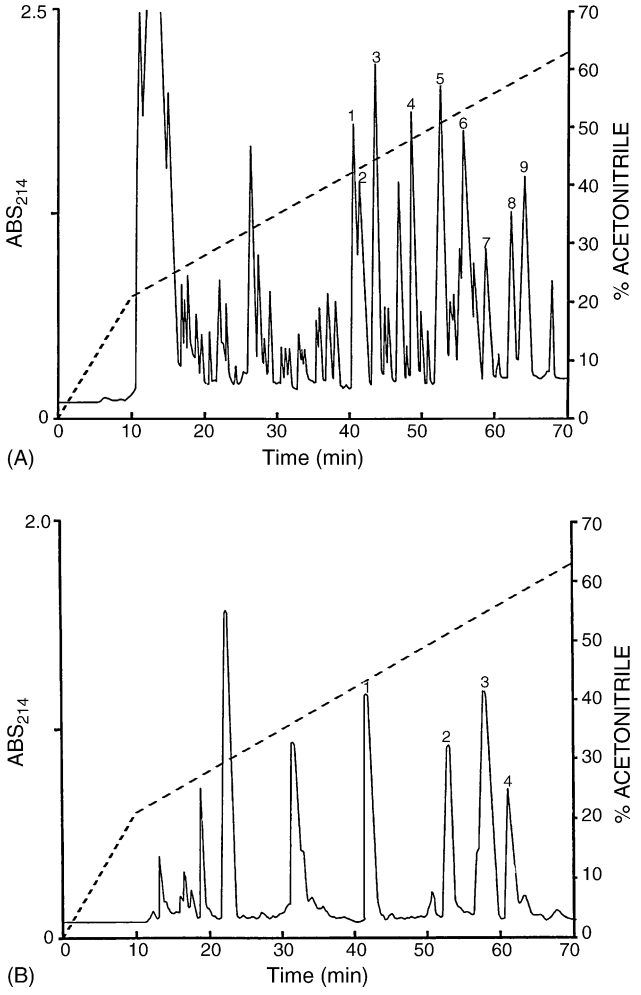

Fig. 1 – Reverse-phase HPLC on a semipreparative Vydac C-18 column of norepinephrine-stimulated skin secretions from (A) *Rana aurora draytonii* and (B) *Rana aurora aurora*. In panel A, peptides in the peaks designated 1–9 were associated with antimicrobial activity and were subjected to further purification. In panel B, peptides in peaks 1–4 were associated with antimicrobial activity and their purification has been described previously [7]. The dashed line shows the concentration of acetonitrile in the eluting solvent.

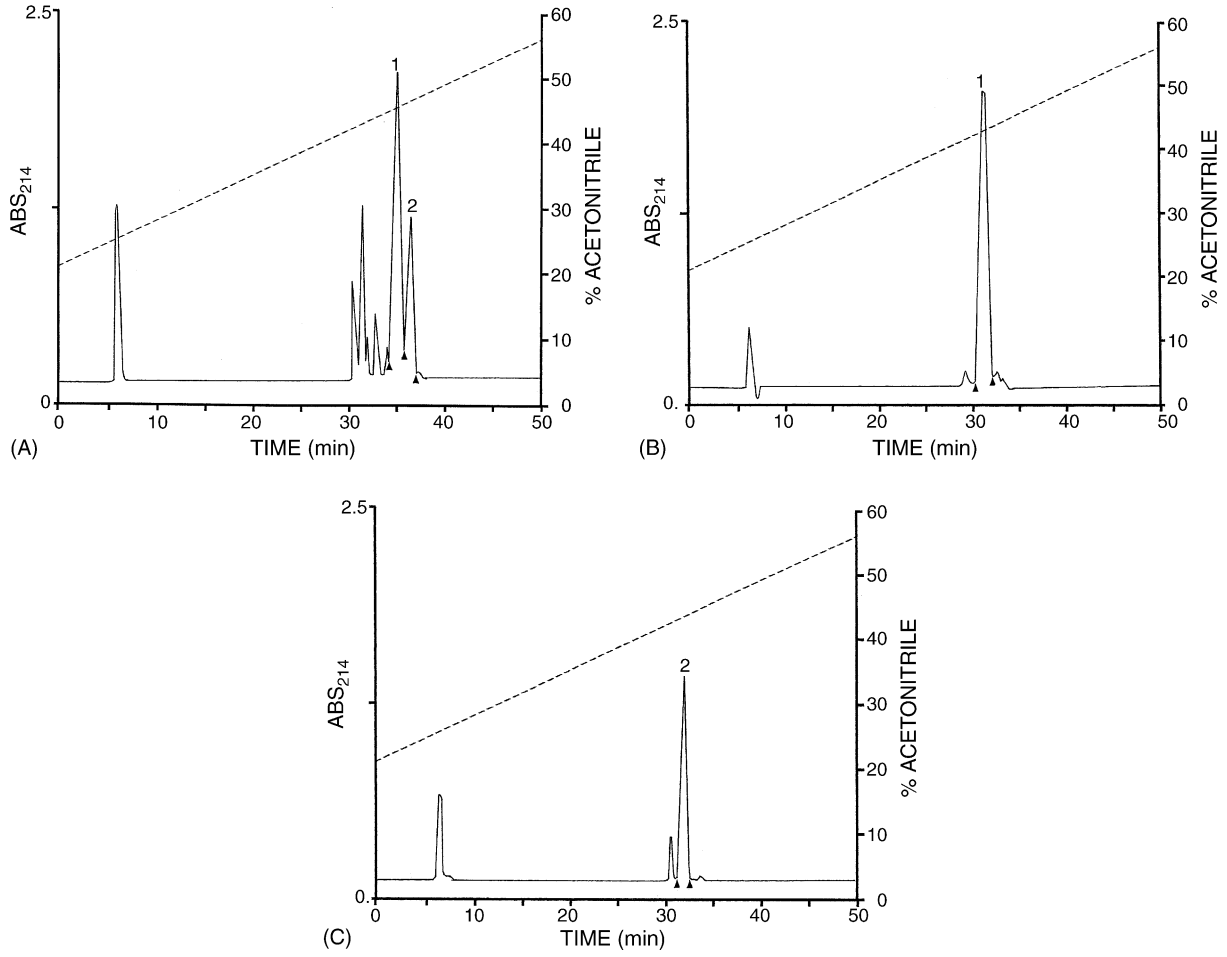

Fig. 2 – Separation of brevinin-1DRb (peak 1) and brevinin-1DRc (peak 2) on a Vydac C-4 column (panel A), and purification to near homogeneity on a Vydac phenyl column of brevinin-1DRb (panel B) and brevinin-1DRc (panel C). The arrowheads show where peak collection began and ended.

conditions of assay, growth inhibitory activity against both *E. coli* and *S. aureus* was associated with peaks 4–8, peaks 1–3 were active only against *E. coli* and peak 9 was active only against *S. aureus*. 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 brevinin-1DRb and brevinin-1DRc on a Vydac C-4 column (Fig. 2A) and final purification of each component on a Vydac phenyl column (Fig. 2B and C).

3.2. *Structural characterization*

The primary structures of the antimicrobial peptides isolated from the skin secretions were established by automated 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. 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. 3). The structural characterization of four peptides with antimicrobial activity (ranatuerin-2AUa, brevinin-1AUa, brevinin-1AUb, and temporin-1AUa) from *R. aurora aurora* skin secretions has been described previously [7].

3.3. *Antimicrobial and hemolytic activities*

As only three juvenile specimens were available to the investigators, it was not possible to obtain sufficient quantities of the purified endogenous peptides to determine their antimicrobial potencies. However, the abilities of synthetic replicates of RV-23, temporin-1DRa, and temporin-1DRb to inhibit the growth of *S. aureus, E. coli,* and *C. albicans*, and to lyse human erythrocytes are quantified in Table 1.

4. Discussion

The present study emphasizes that the mass 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 [1,20,22], in elucidating taxonomic and

Rana aurora draytonii

		M_r (obs)	M_r (calc)
Peptide RV-23	RIGVLLARLPKLFSLFKLMGKKV	2627.0	2626.7
Ranatuerin-2DRa	GIMDTFKGVAKGVAKDLAVKLLDNFKCKITGC	3381.2	3380.8
Ranatuerin-2DRb	GIMDTFKGIAKGVAKNLAGKLLDELKCKMTGC	3351.3	3350.8
Brevinin-1DRa	FLPILAGLAADMLPKVFCSITKKC	2575.4	2576.4
Brevinin-1DRb	FLPILAGLATKIVPKVFCLITKKC	2613.6	2613.6
Brevinin-1DRc	FLPILAGLAAKIVPKVFCLVTKKC	2569.5	2569.5
Brevinin-1DRd	FLPILAGLAAKIVPKVFCLITKKC	2583.6	2583.6
Temporin-1DRa	HFLGTLVNLAKKIL.NH_2	1565.0	1565.0
Temporin-1DRb	NFLGTLVNLAKKIL.NH_2	1542.0	1541.9
Temporin-1DRc	FLPIIASVLSSLL.NH_2	1392.8	1392.9[+]

[+] M_r (calc) refers to the mass of the Na^+ adduct

Rana aurora aurora

Ranatuerin-2AUa	GILSSFKGVAKGVAKNLAGKLLDELKCKITGC	3261.2	3260.8
Brevinin-1AUa	FLPILAGLAAKLVPKVFCSITKKC	2559.5	2559.5
Brevinin-1AUb	FLPILAGLAANILPKVFCSITKKC	2559.4	2559.5
Temporin-1AUa	FLPIIGQLLSGLL.NH_2	1382.7	1382.7

Fig. 3 – 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. aurora draytonii* and *R. aurora aurora*.

phylogenetic relationships between species of ranid frogs. In the laboratory or in the field, skin secretions may be collected from amphibians under non-invasive conditions that do not appear to cause major discomfort or long-term harm to the animals. Mild electrical stimulation [36], injection of norepinephrine into the dorsal lymph sac [28], or immersion of the animal in a solution of norepinephrine (unpublished data) are generally effective. Reverse-phase HPLC provides a rapid method for separation of the antimicrobial peptides in skin secretions and MALDI-TOF mass spectrometry provides a sensitive method for the identification of the major components on the basis of molecular mass.

Application of these techniques to the analysis of norepinephrine-stimulated skin secretions from *R. aurora draytonii* and *R. aurora aurora*, collected under the same conditions and during the same season of the year, demonstrates that both the HPLC elution profiles (Fig. 1) and the mass distribution and amino acid sequences of the antimicrobial peptides (Fig. 3) from both species are quite different. In common with other species belonging to the *Amerana* group of New World ranids, the skins of *R. aurora draytonii* and *R. aurora aurora* synthesize peptides belonging to three families—ranatuerin-2 [15], brevinin-1 [25], and temporin [35]. However, as shown in Table 2, the number of peptides belonging to each family is

Table 1 – Antimicrobial and hemolytic properties of synthetic replicates of peptides isolated from skin secretions of *R. aurora draytoni*

Peptide	*E. coli* MIC (μM)	*S. aureus* MIC (μM)	*C. albicans* MIC (μM)	LC_{50} erythrocytes (μM)
RV-23	2.5	40	80	35
Temporin-1DRa	20	5	40	70
Temporin-1DRb	40	20	80	65

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. draytoni	2	4	2
R. aurora	1	2	1
R. boylii	1	3	2
R. muscosa	1	0	2
R. luteiventris	3	2	2

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

different and characteristic for each species. The amino acid sequences of orthologous peptides are compared in Fig. 4. The variation in amino acid sequences between corresponding *R. aurora draytonii* and *R. aurora aurora* peptides is comparable with the variation in sequences of orthologs from other members of the *Amerana* group of New World ranid frogs (*R. boylii*, *R. muscosa*, *R. luteiventris*). No antimicrobial peptide has the same structure as any other in the species studied. It is proposed, therefore, that the red-legged frogs should be regarded as separate species (*R. aurora* and *R. draytonii*) within the *Amerana* group rather than as conspecific subspecies. The situation is similar to that of the *R. pretiosa* complex which was

Ranatuerin-2

R. a. draytonii	GIMDTFKGVAKGVAKDLAVKLLDNFKCKITGC	100%
R. a. draytonii	GIMDTFKGIAKGVAKNLAGKLLDELKCKMTGC	81%
R. a. aurora	GILSSFKGVAKGVAKNLAGKLLDELKCKITGC	78%
R. muscosa	GLLSSFKGVAKGVAKDLAGKLLEKLKCKITGC	75%
R. muscosa	GIMDSV****KGVAKNLAAKLLEKLKCKITGC	66%
R. boylii	GILSTFKGLAKGVAKDLAGNLLDKFKCKITGC	81%
R. boylii	GIMDSV****KGLAKNLAGKLLDSLKCKITGC	66%
R. luteiventris	GILDSFKGVAKGVAKDLAGKLLDKLKCKITGC	84%
R. luteiventris	GILSSIKGVAKGVAKNVAAQLLDTLKCKITGC	72%

Brevinin-1

R. a. draytonii	FLPILAGLAAKIVPKVFCLITKKC	100%
R. a. draytonii	FLPILAGLATKIVPKVFCLITKKC	96%
R. a. draytonii	FLPILAGLAAKIVPKVFCLVTKKC	96%
R. a. draytonii	FLPILAGLAADMLPKVFCSITKKC	83%
R. a. aurora	FLPILAGLAAKLVPKVFCSITKKC	92%
R. a. aurora	FLPILAGLAANILPKVFCSITKKC	88%
R. boylii	FLPILASLAAKFGPKLFCLVTKKC	79%
R. boylii	FLPILASLAAKLGPKLFCLVTKKC	79%
R. boylii	FLPILASLAATLGPKLLCLITKKC	75%
R. luteiventris	FLPMLAGLAASMVPKLVCLITKKC	83%
R. luteiventris	FLPMLAGLAASMVPKFVCLITKKC	79%

Temporin

Consensus	FLPLIASLL*SKLL	100%
R. a. draytonii	HFLGTLVNLA*KKIL.NH_2	40%
R. a. draytonii	NFLGTLVNLA*KKIL.NH_2	40%
R. a. draytonii	FLPIIASVL*SSLL.NH_2	80%
R. a. aurora	FLPIIGQLL*SGLL.NH_2	73%
R. muscosa	FLPIVGKLL*SGLL.NH_2	67%
R. boylii	FLPIIAKVL*SGLL.NH_2	73%
R. luteiventris	VLPLISMAL*GKLL.NH_2	67%
R. luteiventris	NFLGTLINLA*KKIM.NH_2	33%
R. luteiventris	FLPILINLIHKGLL.NH_2	47%

Fig. 4 – A comparison of the primary structures of peptides belonging to the ranatuerin-2, brevinin-1, and temporin families isolated from New World frogs belonging to the *Amerana* group. Percentage amino acid sequence identities are shown. The shaded residues are conserved. In order to maximize structural similarity, residue deletions denoted by (*) have been introduced in some sequences. The consensus sequence of the temporin family was deduced by Wade [37].

originally regarded as a single species but is now divided into two species with distinct geographical ranges (*R. pretiosa* in the west and *R. luteiventris* in the east) [14].

It is important, however, to point out that species identity is not the only factor determining the distribution of antimicrobial peptides in skin secretions. The environment of the animal plays a role and it has been shown for *Rana esculenta* [23] and *Rana sylvatica* [24] that exposure to bacteria can induce synthesis of particular peptides in the skin. Similarly, it has been shown for *Rana tagoi* that expression of genes encoding antimicrobial peptides is selectively affected by circulating concentrations of thyroid hormones and is subject to seasonal variation (Iwamuro and Conlon, unpublished data).

With few exceptions, the antimicrobial peptides in frog skin secretions are cationic and have the propensity to form an amphipathic α-helical conformation in a membrane-mimetic solvent [4,34]. Peptide RV-23 (designated by its N- and C-terminal amino acids and the number of residues) does not resemble structurally any peptide previously identified in skin secretions of other species belonging to the *Amerana* group. This peptide is strongly cationic (a net charge of +5 at pH 7) and prediction of its secondary structure by the method of Rost and Sander [31] indicates that it has the ability to adopt a stable, extended α-helical conformation. A Schiffer–Edmundsen wheel projection [32] of the RV-23 structure illustrates the amphipathic nature of this α-helix, with the Arg^{1} Arg^{8}, Lys^{11}, and Lys^{22} residues segregating together on one face of the helix and hydrophobic residues segregating on the opposite face. It was not unexpected, therefore, that a synthetic replicate of the peptide showed high potency in inhibiting the growth of the Gram-negative bacteria *E. coli* (MIC = 2.5 μM) but was also quite strongly hemolytic (LC_{50} against human erythrocytes = 35 μM). It has been demonstrated using a series of model α-helical peptides that an increase in cationicity increases antimicrobial potency but an increase in amphipathicity promotes hemolytic activity relative to antimicrobial activity [10]. A BLAST search of the peptides/proteins in the National Center for Biotechnology Information (Bethesda, MD, USA) database reveals that peptide RV-23 shows limited structural similarity to the bee venom peptide, melittin, and the melittin-related peptides previously isolated from the skins of the Eurasian frogs *R. tagoi* [9] and *Rana temporaria* [34] (Fig. 5). These peptides also display potent broad-spectrum antibacterial activity but are strongly hemolytic.

The temporin family, whose members are composed of between 10 and 14 amino acid residues, are among the smallest antimicrobial peptides to be found in nature. Temporins are widely, but not universally, distributed in ranid frogs of both North American and Eurasian origin and more than 50 members of the family have been characterized [5]. These peptides are among the most highly variable of all antimicrobial peptides and no single amino acid residue is invariant. All temporins isolated to-date contain a preponderance of hydrophobic amino acids and are C-terminally α-amidated. Most members of the family contain a single basic residue (generally Lys) giving a charge of +2 at physiological pH, the exception being temporin L (FVQWFSKFLGRIL.NH_2) from *R. temporaria* which bears a net positive charge of +3 [29]. In general, the temporins show greater potencies against Gram-positive bacteria such as *S. aureus* and are either inactive or of low potency against Gram-negative bacteria [5,6]. Temporin L, however, is active against clinically relevant Gram-negative species such as *E. coli* [29]. Temporins-1Da and -1Db isolated in the present study also contain two basic residues and, consistent with previous data [29], these peptides inhibit the growth of *E. coli* with relatively high potency (MIC in the range 20–40 μM) as well as *S. aureus* (MIC 5–20 μM). The therapeutic potential of the many of the temporins is limited by their toxicities against mammalian cells [4] and temporin-1DRa and 1-DRb, like temporin L [29], are appreciably hemolytic towards human erythrocytes (Table 1).

Fig. 5 – A comparison of the primary structures of the melittin-related peptides isolated from the skins of *R. aurora draytonii*, *R. tagoi*, and *R. temporaria* with melittin from the venom of the bee, *Apis mellifera*.

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