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Review

Antimicrobial peptides from the skins of North American frogs

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ABSTRACT

North America is home to anuran species belonging to the families Bufonidae, Eleutherodactylidae, Hylidae, Leiopelmatidae, Ranidae, and Scaphiopodidae but antimicrobial peptides have been identified only in skin secretions and/or skin extracts of frogs belonging to the Leiopelmatidae ("tailed frogs") and Ranidae ("true frogs"). Eight structurally-related cationic α-helical peptides with broad-spectrum antibacterial activity, termed ascaphins, have been isolated from specimens of *Ascaphus truei* (Leiopelmatidae) occupying a coastal range. Characterization of orthologous antimicrobial peptides from *Ascaphus* specimens occupying an inland range supports the proposal that this population should be regarded as a separate species *A. montanus*. Ascaphin-8 shows potential for development into a therapeutically valuable anti-infective agent. Peptides belonging to the brevinin-1, esculentin-1, esculentin-2, palustrin-1, palustrin-2, ranacyclin, ranatuerin-1, ranatuerin-2, and temporin families have been isolated from North American ranids. It is proposed that "ranalexins" represent brevinin-1 peptides that have undergone a four amino acid residue internal deletion. Current taxonomic recommendations divide North American frogs from the family Ranidae into two genera: *Lithobates* and *Rana*. Cladistic analysis based upon the amino acid sequences of the brevinin-1 peptides provides strong support for this assignment.

Contents

1. Introduction

The skin secretions of many, although by no means all, anurans (frogs and toads) contain peptides with antibacterial and antifungal activity. These peptides are stored in granular glands, located mainly in the skin of the dorsal region, that are surrounded by myocytes and innervated by sympathetic fibers. Adrenergic stimulation of the myocytes in response to stress causes compression of the peptide-containing serous cells and discharge of their contents by a holocrine-like mechanism [1]. There are no conserved structural motifs responsible for activity but the vast majority of the frog skin antimicrobial peptides are cationic due to the presence of multiple lysine residues and have the propensity to adopt an amphipathic α-helical conformation in the environment of a phospholipid vesicle or in a membrane-mimetic solvent, such as 50% trifluoroethanol–water [2].

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The peptides have been shown to display potent cytolytic activities *in vitro* against a range of pathogenic microorganisms, including many of those that the animal might be expected to encounter in the wild [3], so that it is reasonable to propose that they represent a component of the system of innate immunity that constitutes the first line of host defense for both vertebrate and invertebrate species [4]. However, the importance of the peptides in the survival strategy of the animal and the extent to which their production provides an evolutionary advantage to the species are not clearly understood. At this time, antimicrobial peptides have been identified in the skins of frogs from species belonging to the Bombinatoridae, Hylidae, Hyperoliidae, Leiopelmatidae, Leptodactylidae, Myobatrachidae, Pipidae, and Ranidae families but several well studied species from the Bufonidae, Ceratophryidae, Dicroglossidae, Microhylidae, Pelobatidae, Pyxicephalidae, Rhacophoridae, and Scaphiopodidae families do not appear to synthesize these peptides [5].

Among those families of anurans found in North America, representatives of the Leiopelmatidae ("tailed frogs") and Ranidae ("true frogs") have been shown to release a diverse array of antimicrobial peptides into their skin secretions. Such peptides have not been detected in skin secretions and/or extracts of those species studied belonging to the Bufonidae, Eleutherodactylidae, Hylidae (subfamily Hylinae), and Scaphiopodidae families [5]. There does not appear to be any obvious correlation between the preferred habitat of the organism and its ability or inability to synthesize dermal antimicrobial peptides. Interpretation of these observations is uncertain but it implies that the synthesis of antimicrobial peptides in the skin may confer advantage to a particular species but is not essential to survival of the organism. This review will describe the structural and biological characteristics of cytolytic peptides found in skin secretions belonging to species from three genera: *Ascaphus* (Leiopelmatidae), *Lithobates* (Ranidae), and *Rana* (Ranidae). The distribution and properties of antimicrobial peptides from frogs of the family Ranidae were the subject of a comprehensive review published in 2004 [6] and so this article will focus on data relating to North American species that have appeared since this time.

2. Taxonomy of North American frogs

The application in recent years of molecular techniques of phylogenetic analysis, particularly comparisons of nucleotide sequences of orthologous genes, has led to quite drastic reappraisals of taxonomic classifications and evolutionary histories of the anurans [7]. Many previously well accepted phylogenetic relationships based upon "classical" criteria, such as morphological characteristics and the fossil record, are being substantially revised. The situation with respect to our understanding of the evolutionary history and phylogenetic relationships of North American frogs is currently in a state of flux [8]. This article follows the taxonomic recommendations and species names proposed in Amphibian Species of the World: an Online Reference. Version 5.2. Electronic Database accessible at http://research.amnh.org/herpetology/amphibia/index.php. American Museum of Natural History, New York, USA [9]. Nomenclature adopted for antimicrobial peptides from frogs of the Ranidae family follows recent guidelines [10].

The tailed frog *Ascaphus* sp. Stejneger, 1899 occupies a uniquely important position in amphibian phylogeny as the most primitive extant anuran [11]. Originally classified alone in the family Ascaphidae as the sister group to the clade of all other living frogs, it is now united with the New Zealand frogs of the genus *Leiopelma* Fitzinger 1861 in the family Leiopelmatidae [9]. Tailed frogs of the genus *Ascaphus* occupy two distinct ranges in the northwest region of North America — the Cascade Mountains and coastal region from British Columbia south to Northern California, and an inland range in the northern Rocky Mountains and the Blue and Wallowa mountains. The ranges are well separated by a zone of reduced rainfall. Although traditionally regarded as a single species, the coastal and inland groups may be distinguished on the basis of morphological characters [12] and, more recently by analysis of nucleotide sequences of mitochondrial cytochrome b and NADH dehydrogenase genes [13,14]. It has been suggested that divergence of the two groups is relatively ancient occurring in response to the rise of Cascade Mountains during the late Miocene (approximately 10 million years ago). The existence of two well differentiated populations has led to the proposal that animals from the inland range should be regarded as a separate species, the Rocky Mountain tailed frog *A. montanus* [13].

Traditionally, the genus *Rana* comprised at least 250 species but subsequent analyses based upon molecular criteria demonstrated that this assemblage did not constitute a monophyletic group [7]. Current taxonomic recommendations have divided the Ranidae into 17 genera [9]. Those North American ranids formerly classified in the Amerana species group (comprising *R. aurora*, *R. boylii*, *R. draytonii*, *R. luteiventris*, *R. muscosa*, and *R. pretiosa*) are retained in the genus *Rana*, which is now restricted to 44 species from Eurasia and North America. The North American bullfrogs formerly classified in the Aquarana species group (comprising *R. catesbeiana*, *R. clamitans*, *R. grylio*, *R. heckscheri*, *R. okaloosae*, *R. septentrionalis*, and *L. virgatipes*) are now united with those in the Pantherana species group, that includes *R. aerolata*, *R. berlandieri*, *R. palustris*, *R. pipiens*, *R. sevosa*, *R. sphenocephala*, *R. sylvatica*, and *R. tarahumarae*, in the genus *Lithobates*. This taxon currently comprises 49 species from North, Central and South America to southern Brazil. Table 1 summarizes the currently accepted species names of frogs from which antimicrobial peptides have been isolated that were previously classified in the genus *Rana*. It should be pointed out that this division of North American ranids into the two genera *Rana* and *Lithobates* has been subject to criticism [15].

Table 1
Distribution of antimicrobial peptides in the skins of North American species of the genera *Rana* and *Lithobates*

Original name	Reclassified	Antimicrobial peptides
R. aurora	stet	Brevinin-1 (2), Ranatuerin-2 (1), Temporin (1)
R. boylii	stet	Brevinin-1 (3), Ranatuerin-2 (2), Temporin (1)
R. cascadae	stet	Brevinin-1 (1), Ranatuerin-2 (1), Temporin (4)
R. draytonii	stet	Brevinin-1 (4), Ranatuerin-2 (2), Temporin (3)
R. luteiventris	stet	Brevinin-1 (2), Ranatuerin-2 (2), Temporin (3)
R. muscosa	stet	Ranatuerin-2 (2), Temporin (1)
R. aerolata	*L. aerolatus*	Brevinin-1 (1), Esculentin-1 (3), Palustrin-2 (1) Ranacyclin (2), Ranatuerin-2 (2), Temporin (1)
R. berlandieri	*L. berlandieri*	Brevinin-1 (6), Esculentin-2, Ranatuerin-2 (1)
R. catesbeiana	*L. catesbeianus*	Brevinin-1 (2), Ranatuerin-1 (1) Ranatuerin-2 (3) Temporin (5)
R. clamitans	*L. clamitans*	Brevinin-1 (2), Ranatuerin-1 (1) Ranatuerin-2 (2), Temporin (5)
R. grylio	*L. grylio*	Brevinin-1 (1), Ranatuerin-1 (2) Ranatuerin-2 (1) Temporin (4)
R. heckscheri	*L. heckscheri*	Brevinin-1 (1), Ranatuerin-2 (1), Temporin (1)
R. okaloosae	*L. okaloosae*	Brevinin-1 (1), Palustrin-2 (1), Ranatuerin-2 (1) Temporin (2)
R. palustris	*L palustris*	Brevinin-1 (3), Esculentin-1 (3), Esculentin-2 (1) Palustrin-1 (4), Palustrin-2 (3), Ranacyclin (3) Ranatuerin-2 (6), Temporin (1)
R. pipiens	*L. pipiens*	Brevinin-1 (4), Esculentin-2 (1), Ranatuerin-2 (1) Ranacyclin (1), Temporin (1)
R. septentrionalis	*L. septentrionalis*	Brevinin-1 (4), Brevinin-2-related (1) Ranatuerin-2 (2) Temporin (3)
R. sevosa	*L. sevosus*	Brevinin-1 (1), Esculentin-1 (2), Esculentin-2 (1) Palustrin-2 (1), Ranatuerin-2 (2)
R. sphenocephala	*L. sphenocephalus*	Brevinin-1 (3)
R. sylvatica	*L. sylvaticus*	Brevinin-1 (1)
R. tarahumarae	*L. tarahumarae*	Brevinin-1 (3) Ranatuerin-2 (3)
R. virgatipes	*L. virgatipes*	Brevinin-1 (1), Brevinin-2-related (2), Ranatuerin-2 (3) Temporin (3)

The values in parentheses show the number of paralogs identified in each species. The term stet means no change in the species name.

3. Antimicrobial peptides from North American frogs of the Leiopelmatidae family

A comparison of the primary structures of the antimicrobial peptides from specimens of *Ascaphus* from the coastal and inland ranges supports the assertion that the two populations of the tailed frog should be recognized as a distinct species [13,20]. Eight peptides with broad-spectrum antimicrobial activity, termed ascaphin 1–8, were isolated from norepinephrine-stimulated skin secretions of *A. truei* (coastal range) [16]. As shown in Fig. 1, the peptides are structurally similar to each other, suggesting an origin that involves multiple duplications of an ancestral gene [17], but they do not resemble closely any antimicrobial peptide isolated from the skins of other frog species. The ascaphins do, however, display limited sequence similarity with the cationic, amphipathic α-helical peptides pandinin 1 [18] and opistoporin 1[19] isolated from the venoms of African scorpions but this may be fortuitous rather than indicating an evolutionary relationship. Ascaphin-1, -3, -5, -7 isolated from tailed frogs from the inland range (designated M in Fig. 1) show differences in amino acid compared with the corresponding peptides from *A. truei* thereby supporting the assignment of this population to the separate species *A. montanus* [19]. Orthologs of ascaphin-2, -6 and -8 were not detected in the secretions from the inland range frogs.

Ascaphin-8 shows possibilities for development into a therapeutically valuable anti-infective agent. Circular dichroism (CD) spectra demonstrate that the peptide adopts an amphipathic α-helical conformation in a membrane-mimetic solvent such as 50% trifluoroethanol–water [20]. Ascaphin-8 inhibits with relatively high potency (minimum inhibitory concentration MIC ≤ 25 μM) the growth of a range of clinical isolates of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* as well as a group of miscellaneous ESBL-producing strains (*Citrobacter*, *Salmonella*, *Serratia*, *Shigella* spp.) [21]. Unexpectedly, ESBL-producing *Proteus mirabilis* strains were susceptible to ascaphin-8 (MIC = 12.5–25 μM) although non-ESBL isolates of this organism were resistant (MIC > 100 μM). The therapeutic potential of ascaphin-8, especially for systemic application, is limited by toxicity against mammalian cells (LC_{50} against human erythrocytes = 55 μM). Analogs containing amino acid substitutions at Ala^{10}, Val^{14}, and Leu^{18} in ascaphin-8 by either L-Lys or D-Lys display increased cationicity while maintaining amphipathicity and these peptides retain antimicrobial activity against Gram-negative and Gram-positive bacteria and against the opportunistic yeast pathogen, *Candida albicans* but showed appreciably reduced toxicities (>10-fold) against human erythrocytes, HepG2 hepatoma-derived cells, and L929 fibroblasts [22].

Ascaphin-1	GFRDVLKGAAKAFVKTVAGHIAN.NH_2
Ascaphin-1M	----------E----------.NH_2
Ascaphin-2	GFRDVLKGAAKQFVKTVAGHIANI
Ascaphin-3	GFRDVLKGAAKAFVKTVAGHIANI
Ascaphin-3M	---E--------------------
Ascaphin-4	GFKDWIKGAAKKLIKTVAANIANQ
Ascaphin-4M	------------------S-----
Ascaphin-5	GIKDWIKGAAKKLIKTVASHIANQ
Ascaphin-5M	----------T------------
Ascaphin-6	GFKDWIKGAAKKLIKTVASSIANE
Ascaphin-7	GFKDWIKGAAKKLIKTVASSIANQ
Ascaphin-7M	-------S----------N----
Ascaphin-8	GFKDLLKGAAKA LVKTVLF.NH_2

Fig. 1. A comparison of the primary structures of peptides belonging to the ascaphin family from *A. truei* (coastal range) and from *A. montanus* (inland range, designated M). (—) denotes those amino acids that are conserved in the peptides.

4. Antimicrobial peptides from North American frogs of the Ranidae family

4.1. Distribution and molecular heterogeneity

Skin secretions of North American ranid frogs produce a diverse array of antimicrobial peptides that may be grouped together in families on the basis of limited structural similarity. The degree of molecular heterogeneity among peptides within a particular family is appreciable with a peptide from one species rarely being found with an identical amino acid sequence in another, even when species are quite closely related to each other phylogenetically. Nucleotide sequence analysis of cDNAs encoding the biosynthetic precursors of these peptides has shown that the structural organization of the precursors is quite similar, comprising a signal peptide sequence, an acidic N-terminal spacer peptide region, and the antimicrobial peptide at the C-terminus of the precursor [17]. Despite the fact that the primary structures of antimicrobial peptides from the various families show very little similarity to each other, the amino acid sequences of the signal peptide and acidic pro-region have been remarkably well conserved in the different precursors. It was proposed, therefore, that the families share a common evolutionary origin having arisen from multiple duplications of an ancestral gene during radiation of the species, and within individual species [23]. This conclusion is supported by identification of multiple copies of genes encoding antimicrobial peptides of the brevinin-1 family in the genomes of individual specimens of the leopard frogs *L. pipiens*, *L. chiricahuensis*, and *L. sphenocephalus* [24]. Allelic variation is low compared with inter-species divergence and an excess of nonsynonymous substitutions in the antimicrobial peptide coding region is indicative of positive natural selection.

At this time, at least 13 well-established peptide families have been identified in the skins of ranid frogs and may be listed as brevinin-1, brevinin-2, esculentin-1, esculentin-2, japonicin-1, japonicin-2, nigrocin-2, palustrin-1, palustrin-2, ranacyclin, ranatuerin-1, ranatuerin-2, and temporin (Table 1) [6]. Those families containing members that have been isolated from North American species are underlined. However, there are no well-defined amino acid motifs that determine antimicrobial activity so that unambiguous assignment of a newly identified peptide to a particularly family can be difficult or somewhat arbitrary. Unfortunately, peptides from various species that show clear structural similarity to each other, indicative of a common evolutionary origin, are often given completely different names by the investigator who first identified them so that there is a clear need for a universally accepted terminology [10].

As well as a source of compounds with therapeutic potential, the amino acid sequences of antimicrobial peptides in skin secretions may be used to infer taxonomic and phylogenetic relationships between species of ranid frogs [6]. For example, analysis of the distribution and primary structures of dermal peptides from the Northern red-legged frog [25] and the California red-legged frog [26] provides support for the proposal that these anurans should be regarded as separate species (*R. aurora* and *R. draytonii*) rather than conspecific subspecies (*Rana aurora aurora* and *Rana aurora draytonii*). Similar analysis of the structures of antimicrobial peptides from the N. American bullfrogs (Aquarana) indicates a sister-group relationship between *L. heckscheri* and *L. grylio* and a close, but less well defined, phylogenetic relationship between *L. okaloosae* and *L. clamitans* [27]. In general, cladograms based upon the amino acid sequences of antimicrobial peptides show a moderate to high degree of consistency with those based upon comparisons of the nucleotide sequences of mitochondrial and ribosomal genes [28,29].

4.2. Brevinin-1 peptides

As shown in Table 1, peptides of the brevinin-1 family, first identified in the skin of the Asian frog *R. brevipoda porsa* [30] (now reclassified as *Pelophylax porosus*), are very widely distributed among the North American ranids having been detected in skin secretions and/or skin extracts of all species investigated to-date except for *R. muscosa* [31]. The inability to detect brevinin-1 peptides in this species does not necessary mean that the gene is not expressed as it is possible that a peptide with low antimicrobial potency would be missed. The problem of assigning peptides to particular family is well illustrated by the relationship between brevinin-1 and ranalexin. Ranalexin was first isolated from an extract of whole *L. catesbeianus* tadpoles [32] and its distribution is restricted to those North American species formerly classified within the Aquarana species group [8]. Up to this time, ranalexin peptides have never been detected in the skins of ranid frogs from other species groups and so

L. aerolatus	FLPLVR*VAAKILPSVFCAISKRC
L. berlandieri a	FLPFIAGMAAKFLPKIFCAISKKC
L. berlandieri b	FLPAIAGMAAKFLPKIFCAISKKC
L. berlandieri c	FLPFIAGVAAKFLPKIFCAISKKC
L. berlandieri d	FLPAIAGVAAKFLPKIFCAISKKC
L. berlandieri e	FLPAIVGAAAKFLPKIFCVISKKC
L. berlandieri f	FLPFIAGMAANFLPKIFCAISKKC
L. catesbeianus a	FL****GGLIKIVPAMICAVTKKC
L. catesbeianus b	FLPFIARLAAKVFPSIICSVTKKC
L. clamitans a	FL****GGLMKAFPALICAVTKKC
L. clamitans b	FL****GGLMKAFPAIICAVTKKC
L. grylio	FL****GGLMKIIPAAFCAVTKKC
L. heckscheri	FL****GGLIKIIPAAFCAVTKKC
L. okaloosae	FM****GGIMKAIPAMICAMTKKC
L. palustris a	FFPNVASVPGQVLKKIFCAISKKC
L. palustris b	FLPLIAGLAANFLPKIFCAITKKC
L. palustris c	FLPVIAGVAAKFLPKIFCAITKKC
L. pipiens a	FLPIIAGVAAKVFPKIFCAISKKC
L. pipiens b	FLPIIAGIAAKVFPKIFCAISKKC
L. pipiens c	FLPIIASVAAKVFSKIFCAISKKC
L. pipiens d	FLPIIASVAANVFSKIFCAISKKC
L. pipiens e	FLPIIASVAAKVFPKIFCAISKKC
L. septentrionalis a	FFPIIAGMAAKLIPSLFCKITKKC
L. septentrionalis b	FLPIIAGMAAKVI****CAITKKC
L. septentrionalis c	FLPIIASVAAKLIPSIVCRITKKC
L. septentrionalis d	FFPIIAGMAAKVI****CAITKKC
L. sevosus	FLPLVR*GAAKLIPSVVCAISKRC
L. sphenacephalus a	FLPAIVGAAGQFLPKIFCAISKKC
L. sphenacephalus b	FLPAIVGAAAKFLPKIFCAISKKC
L. sphenacephalus c	FFPIVAGVAGQVLKKIYCTISKKC
L. sylvaticus	FLPVVAGLAAKVLPSIICAVTKKC
L. tarahumarae a	FLPVIAGIAANVLPKLFCKLTKRC
L. tarahumarae b	FLPFIASMAAKLVPKLVCAITKKC
L. tarahumarae c	FLPVLAGIAANVLPTLICKLTRRC
L. virgatipes	FL****GGLFKLVPSVICAVTKKC
R. aurora a	FLPILAGLAAKLVPKVFCSITKKC
R. aurora b	FLPILAGLAANILPKVFCSITKKC
R. boylii a	FLPILASLAAKFGPKLFCLVTKKC
R. boylii b	FLPILASLAAKLGPKLFCLVTKKC
R. boylii b	FLPILASLAATLGPKLLCLITKKC
R. cascadae	FLPILAGLAAKIVPKLFCLATKKC
R. draytonii a	FLPILAGLAAKIVPKVFCLITKKC
R. draytonii b	FLPILAGLATKIVPKVFCLITKKC
R. draytonii c	FLPILAGLAAKIVPKVFCLVTKKC
R. draytonii d	FLPILAGLAADMLPKVFCSITKKC
R. luteiventris a	FLPMLAGLAASMVPKLVCLITKKC
R. luteiventris b	FLPMLAGLAASMVPKFVCLITKKC

Fig. 2. Primary structures of peptides of the brevinin-1 family isolated from species of North American frogs belonging to the genera *Rana* and *Lithobates*. Those sequences underlined were originally designated ranalexins. (*) denotes deletion of an amino acid residue from a putative ancestral peptide.

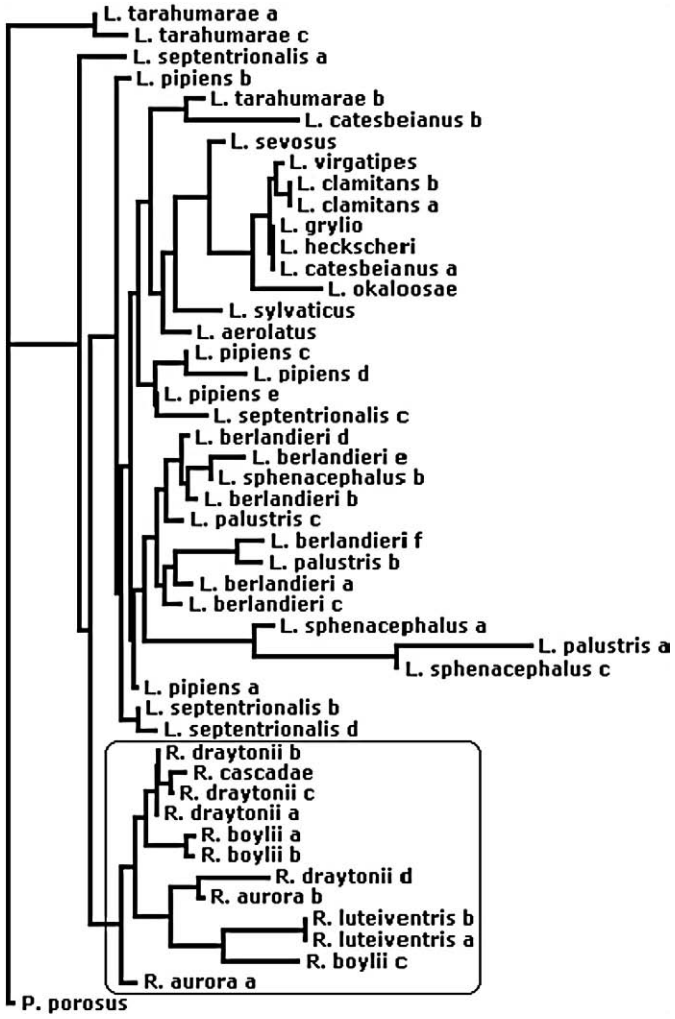

Fig. 3. A phylogenetic tree based upon the amino acid sequences of the brevinin-1 peptides isolated from the skins of North American frogs that are shown in Fig. 2. Cladistic analysis was performed using the Phylogeny Inference Program package, PHYLIP, version 3,57c (J. Felsenstein, University of Washington, Seattle WA, 1993). The stability of the tree was tested by bootstrap resampling analysis of 1000 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 the corresponding 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 brevinin-1 from the Asian frog *P. porosus* (FLPVLAGIAAKVVPALFCKITKKC) was used as outgroup.

the question arises as to whether ranalexin is encoded by a separate gene that arose relatively late in evolution within the common ancestor of the Aquarana or whether it represents an ortholog of another more widely distributed antimicrobial peptide. As shown in Fig. 2, the amino sequences of the ranalexins show strong structural similarity to members of the brevinin-1 family from other North American species including the presence of the strongly conserved Phe[1], Lys[11], Pro[14], Cys[18], Lys[22], Lys[23], and Cys[24] residues. Structural similarity is maximized if it is assumed that the genes encoding the ranalexins have undergone a deletion of a region encoding a contiguous tetrapeptide. Consequently, the term “ranalexin” becomes superfluous and should no longer be used. It is significant that the skin of a seventh member of the Aquarana species group, *L. septentrionalis* contains brevinin-1 peptides from which a different tetrapeptide has been deleted (Fig. 2) [33]. By the same token, molecular cloning studies have demonstrated that the melittin-related peptides found in the skin of *Rana draytonii* and in the skins of certain Japanese brown frogs and are not evolutionarily related to

the peptides in bee venom and they have also been tentatively assigned to the brevinin-1 family [34].

Previous studies involving ranid frogs have shown that that the amino acid sequences of brevinin-1 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 [5,35]. A neighbor-joining analysis based upon the amino acid sequences of the known brevinin-1 peptides isolated from North American frogs (Fig. 3) provides strong support for assignment of species into the two genera *Rana* and *Lithobates*. All species in the genus *Rana*, formerly classified in the Amerana group [8,28], segregate together in a single clade denoted by the box in Fig. 3 that is distinct from the clade containing the species in the genus *Lithobates*. Placement of the ranalexin sequences from bullfrogs formerly classified within the Aquarana species group [8,29] within a larger clade containing brevinin-1 sequences supports the assertion that the ranalexins represent brevinin-1 peptides that have undergone a four amino acid residue internal deletion (Fig. 2).

Among the brevinin-1 peptides isolated from North American species, brevinin-1BYa from the foothill yellow-legged frog *R. boylii* has been the most extensively studied [36]. A synthetic replicate of the peptide showed growth inhibitory activity (minimum inhibitory concentration MIC ≤ 10 μM) against a range of reference strains of Gram-positive and Gram-negative bacteria, including *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, and *Streptococcus* group B, against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) (MIC = 2.5 μM), and against reference strains and clinical isolates of the opportunistic yeast pathogens *C. albicans*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis* (MIC ≤ 10 μM). However, the therapeutic potential of the peptide, especially for systemic applications, is restricted by its high hemolytic activity against human erythrocytes (LD_{50} = 10 μM). Replacement of the cysteine residues in brevinin-1BYa by serine produced an acyclic analog with eight-fold reduced hemolytic activity that retained high potency against Gram-positive bacteria, including strains of MRSA (MIC = 5 μM) suggesting that the peptide represents a candidate for drug development, particularly for topical applications against antibiotic-resistant microorganisms. The broad-spectrum antimicrobial activity of "ranalexin" is similar to that of members of the brevinin-1 family [6].

4.3. Ranatuerin-2 peptides

Members of the ranatuerin-2 family of peptides, first identified in the skin of the bullfrog *L. catesbeianus* [37], are present in skin secretions of most species of ranid frogs of North American origin studied to-date (Table 1). The family has a more restricted distribution in Eurasian species [6]. A C-terminal cyclic hexapeptide domain, rather than the more common heptapeptide, characterizes the peptide. Its

L. aerolatus a	GLM*DTVKNAAKNLA****GQLLDTIKCKMTGC
L. aerolatus b	GIL*DTIKNAAKTVA****VGLLEKIKCKMTGC
L. berlandieri	GLL*DTIKGVAKTVA****ASMLDKLKCKISGC
L. catesbeianus a	GLFLDTLKGAAKDVA****GK*LEGLKCKITGCKLP
L. catesbeianus b	GVFLDTLKGLAGKM********LESLKCKIAGCKP
L. catesbeianus c	GFL*DIIKNLGKTFA****GHMLDKIKCTIGTCPPSP
L. clamitans a	GLFLDTLKGAAKDVA****GKLLEGLKCKIAGCKP
L. clamitans b	GLFLDTLKGLA********GKLLQGLKCIKAGCKP
L. grylio	GLLLDTLKGAAKDIA****GIALEKLKCKITGCKP
L. heckscheri	GLFLDTLKGAAKDIA****GIALEKLKCKITGCKP
L. okaloosae	GLFVDTLKGLA********GKMLESLKCKIAGCKP
L. palustris a	GIM*DTVKNVAKNLA****GQLLDKLKCKITAC
L. palustris b	GIM*DTVKNAAKDLA****GQLLDKLKCRITGC
L. palustris c	GLL*DTIKNTAKNLA****VGLLDKIKCKMTGC
L. palustris d	GIM*DSVKNVAKNIA****GQLLDKLKCKITGC
L. palustris e	GIM*DSVKNAAKNLA****GQLLDTIKCKITAC
L. palustris f	GIM*DTVKNAAKDLA****GQ*LDKLKCRITGC
L. pipiens	GLM*DTVKNVAKNLA****GHMLDKLKCKITGC
L. septentrionalis a	GLFLNTVKDVAKDVAKDVAGKLLESLKCKITGCKS
L. septentrionalis b	GLFLNTVKDVAKDVAKDVAGKLLESLKCKITGCKP
L. sevosus a	AIM*DTIKDTAKTVA****VGLLNKLKCKITGC
L. sevosus b	GIM*DTIKDTAKTVA****VGLLNKLKCKITGC
L. tarahumarae a	GIM*DSIKGAAKEIA****GHLLDNLKCKITGC
L. tarahumarae b	GIL*DTLKNVAKNVA****AGLLDNIKCKITGC
L. tarahumarae c	GIF*DTIKNVAKNMA****AGLLDNIKCKITGC
L. virgatipes a	GVFLDTLKGVGKDAA****VKLLEALQCKFGVCKN
L. virgatipes b	GVFLDALKGVGKGVA****VSLLNGLKCKLGVC
L. virgatipes c	GVFLNTIKEVGKDAA****VKLLEALQCKFGVCKT
R. aurora	GIL*SSFKGVAKGVAKNLAGKLLDELKCKITGC
R. boylii a	GIL*STFKGLAKGVAKDLAGNLLDKFKCKITGC
R. boylii b	GIM*DSVKGLAKNLA****GKLLDSLKCKITGC
R. cascadae	GIL*SSFKGVAKGVAKDLAGKLLETLKCKITGC
R. draytonii a	GIM*DTFKGVAKGVAKDLAVKLLDNFKCKITGC
R. draytonii b	GIM*DTFKGIAKGVAKNLAGKLLDELKCKMTGC
R. luteiventris a	GIL*DSFKGVAKGVAKDLAGKLLDKLKCKITGC
R. luteiventris b	GIL*SSIKGVAKGVAKNVAAQLLDTLKCKITGC
R. luteiventris c	GIL*SSFKGVAKGVAKDLAGKLLDTLKCKITGC
R. luteiventris d	GIL*SSIKGVAKNVA****AQLLDTLKCKITGC
R. muscosa a	GLL*SSFKGVAKGVAKDLAGKLLEKLKCKITGC
R. muscosa b	GIM*DSVKGVAKNLA****AKLLEKLKCKITGC

Fig. 4. Primary structures of peptides of the ranatuerin-2 family isolated from species of North American frogs belonging to the genera *Rana* and *Lithobates*. (*) denotes deletion of an amino acid residue.

primary structure has been poorly conserved among peptides from North American frogs with several residue deletions and only the cysteine residues invariant (Fig. 4). Ranatuerin-2 is also a peptide whose amino acid sequence has been used in phylogenetic analyses to infer evolutionary relationships between species [6,27,38]. As shown in Fig. 5, a neighbor-joining analysis based upon the amino acid sequences of the known ranatuerin-2 peptides isolated from North American frogs (Fig. 4) provides some support for the division of species into *Rana* and *Lithobates* but evidence is less convincing than in the case of brevinin-1 (Fig. 3). Ranatuerin-2 peptides from frogs from the genus *Rana* segregate with a single clade, denoted by the box in Fig. 5, but embedded within this clade are sequences from *L. palustris* and *L. tarahumarae*. Similarly, the well defined clade containing all the sequences from the *Lithobates* species formerly classified in the Aquarana group also contains a sequence from *L. berlandieri*.

The marked variation in the amino acid sequence of ranatuerin-2 is matched by a correspondingly wide variability in observed antimicrobial specificities and potencies and in hemolytic activities [6]. The potent broad-spectrum antimicrobial activity but relatively weak hemolytic activity of ranatuerin-2CSa, first isolated from skin secretions of the Cascades frog, *Rana cascadae* [38], suggests that the peptide may have the potential for antibacterial drug development. The solution structure of ranatuerin-2CSa has been investigated by proton NMR spectroscopy and molecular modelling [39]. In aqueous solution, the peptide lacks secondary structure but, in a trifluoroethanol–water solvent mixture, the peptide adopts a stable conformation that is characterized by a full length helix-turn-helix motif between residues Ile^{2}-Leu^{21}, Leu^{22}-Leu^{25} and Lys^{26}-Thr^{30} respectively.

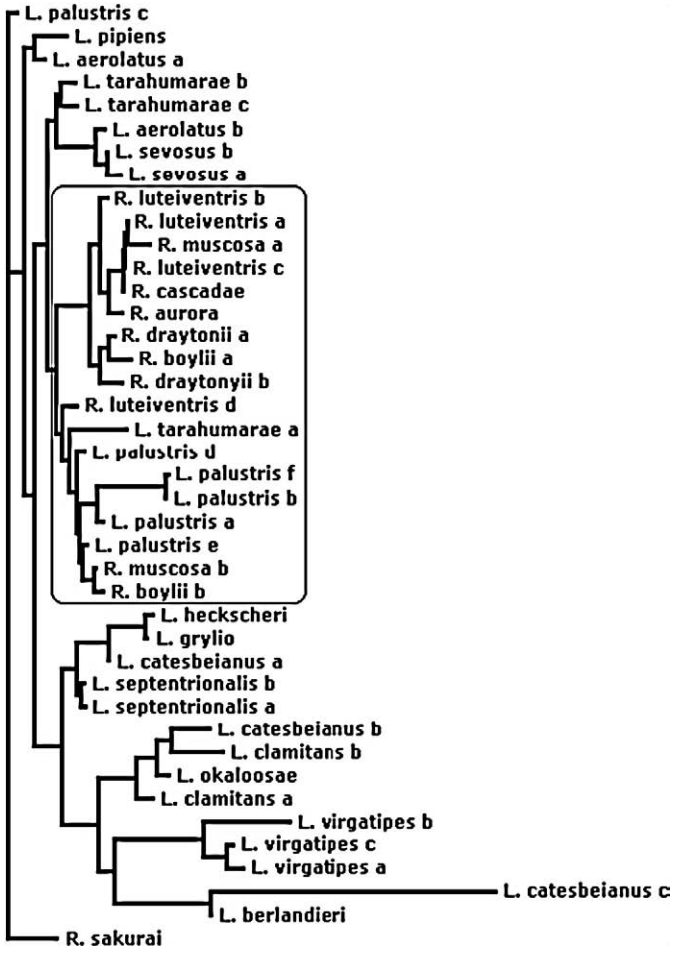

Fig. 5. A phylogenetic tree based upon the amino acid sequences of the ranatuerin-2 peptides isolated from the skins of North American frogs that are shown in Fig. 4. Cladistic analysis was carried out as described in the legend to Fig. 3. The amino acid sequence of ranatuerin-2 from the Asian frog *R. sakuraii* (GLLDAIKDTAQNLFAN VLDKIKCKFTKC) was used as outgroup.

Lithobates	***Rana***
L. catesbeianus	*R. boylii*
FLP*IASLLGKYL	FLPIIAKVLSGLL
FISAIASMLGKFL	
FLSAIASMLGKFL	*R. muscosa*
FISAIASFLGKFL	FLPIVGKLLSGLL
FLFPLITSFLSKVL	
	R. aurora
L. grylio	FLPIIGQLLSGLL
SILPTIVSFLSKVF	
SILPTIVSFLSKFL	*R. draytonii*
SILPTIVSFLTKFL	HFLGTLVNLAKKIL
FILPLIASFLSKFL	NFLGTLVNLAKKIL
L. clamitans	*R. luteiventris*
FLPFLAKILTGVL	VLPLISMALGKLL
FLPLFASLIGKLL	NFLGTLINLAKKIM
FLPFLASLLTKVL	FLPILINLIHKGLL
FLPFLASLLSKVL	
FLPFLATLLSKVL	
L. septentrionalis	
FLSAITSILGKFF	
FLSAITSLLGKLL	
FLSAITSILGKLF	
L. virgatipes	
FLSSIGKILGNLL	
FLSIIAKVLGSLF	
FLPLVTMLLGKLF	
L. heckscheri	
SIFPAIVSFLSKFL	
L. okaloosae	
FLPFLKSILGKIL	
FLPFFASLLGKLL	
L. aerolata	
FLPIVGRLISGLL	
L. palustris	
FLPLVGKILSGLI	
L. pipiens	
FLPIVGKLLSGLL	

Fig. 6. Primary structures of peptides of the temporin family isolated from species of North American frogs belonging to the genera *Rana* and *Lithobates*. All peptides are C-terminally α-amidated.

4.4. Temporin peptides

Peptides of the temporin family are widely distributed among species of Eurasian and New World ranids and approaching 100 different members of the family have been identified to-date [40]. The primary structures of those temporins isolated from North American frogs are shown in Fig. 6. Because of their small size (10–21 amino acid residues) and ease of synthesis, the temporins have received attention as lead compounds for development into therapeutically valuable anti-infective agents [41]. Among the temporin peptides from

N. American ranids, the antimicrobial properties of temporin-DRa, first isolated from norepinephrine-stimulated skin secretions of the California red-legged frog *R. draytonii* [26], and temporin-Va from the carpenter frog, *L. virgatipes* [42] have been studied in most detail. Unlike many of the more hydrophobic members of the family eg temporin-Vc, these peptides are very soluble in aqueous media. Most temporins show growth-inhibitory activity only against Gram-positive bacteria but temporin-DRa and temporin-Va are atypical in displaying activity against reference strains of several clinically relevant Gram-negative species (*E. coli*, *K. pneumoniae*, *E. cloacae*. *P. aeruginosa*, *Salmonella typhimurium*) and against the opportunistic yeast pathogen *C. albicans* as well as multidrug-resistant strains of *S. aureus* (MRSA) [43]. In addition to broad-spectrum activity against aerobic bacteria, temporin-DRa and temporin-Va show high potency (MIC ≤ 12.5 μM) against reference strains and clinical isolates of the Gram-positive anaerobes *Propionibacterium acnes* and *Clostridium tertium* and were active (MIC ≤ 50 μM) against the Gram-positive cocci *Poststreptococcus asacharolyticus* and *Poststreptococcus anaerobius* and the Gram-negative bacillus *Provotella melaninogenica* [43].

The therapeutic potential of temporin-DRa is restricted by its significant hemolytic activity against human erythrocytes (LD_{50} = 70 μM). A structure-activity study has demonstrated that analogs containing the substitutions (Val^7 → L-Lys), (Thr^5 → D-Lys) and (Asn^8 → D-Lys) retained the high solubility and potent, broad spectrum antimicrobial activity of the naturally occurring peptide but were appreciably (up to 10-fold) less hemolytic [44]. Similarly, substitution of Ile^{13} by α-aminoisobutyric acid (Aib) produces a 4-fold increase in therapeutic index (ratio of LC_{50} against erythrocytes to MIC against microorganisms) [45], identifying these peptides as compound with potential for development as anti-infective agents. The data suggest a strategy that involves selective increases in cationicity that maintain amphipathicity but increase Φ (the angle subtended by the charged residues) in order to transform naturally-occurring temporins into non-toxic analogs with therapeutic utility. Increases in hydrophobicity and helicity generally increase cytolytic activity against mammalian cells. This conclusion is consistent with several studies involving other naturally occurring and synthetic antimicrobial peptides (reviewed in [44]).

In addition to their abilities to inhibit growth of microorganisms, temporin-Vb from *L. virgatipes* [42] and temporin-DRb from *R. draytonii* [26], in the concentration range 10^{-8}–10^{-6} M, produced significant ($p<0.05$) and dose-dependent stimulatory effects on insulin secretion from clonal rat BRIN-BD11 insulinoma-derived cells [46]. This increase in the rate of insulin release was not associated with an increased release of lactate dehydrogenase indicating that the integrity of the plasma membrane was maintained. The observation raises the possibility that the peptides may be developed into agents with therapeutic potential for treatment of patients with Type 2 diabetes.

4.5. Ranacyclin-related peptides

The term ranacyclin was first used to describe two 17-amino acid residue peptides with antibacterial and antifungal activities synthesized in the skins of the Eurasian frogs *R. temporaria* (ranacyclin T) and *R. esculenta* (now reclassified as a hybridogenetic complex between *Pelophylax lessonae* and *P. ridibundus*) (ranacyclin E) [47]. A comparison of their primary structures reveals a high degree of sequence identity with peptides previously identified in skin secretions of the North American frogs *L. pipiens* (termed peptide LR) [48] and *L. palustris* [49], and limited similarity with peptides from *R. aerolata* [50] suggesting that the ranacyclins constitute a peptide family with a common origin (Fig. 7). Ranacyclins E and T and peptide LR are exceptional among frog skin antimicrobial peptides in that they adopt a predominantly random coil conformation even in a membrane-mimetic solvent such as 1% sodium dodecylsulfate solution, as demonstrated by CD and Fourier Transform Infrared (FTIR) spectroscopy. The ranacyclins bind and insert into both zwitterionic (phosphatidylcholine/cholesterol) and negatively charged (phosphatidylglycerol/phosphatidylethanolamine) model membrane vesicles suggesting that the primary interaction is with the hydrophobic core of the cell membrane rather than the more usual electrostatic interaction of the peptide with the lipid head group. A two-step mode of action was proposed with the peptides first aligning parallel to the outer membrane surface bound by predominantly hydrophobic interactions and subsequently, at higher peptide concentrations, inserting into the hydrophobic core of the membrane bilayer to form transmembrane pores [47].

Ranacyclin

R. temporaria	GALRGCW TKSYPPKPCK
P. lessonae/ridibundus	SAPRGCW TKSYPPKPCK
L. pipiens	LVRGCW*TKSYPPKPCFVR
L. palustris a	SVIGCWKTKSIPPRPCFFK
L. palustris b	LIRGCW*TKSIPPK*CPLV
L. palustris c	SVIGCW*TKSIPPRPCFV
L. aerolata a	YLRRCWK*KPNLGIVCS
L. aerolata b	YLRGCWK*KPNLGIVCS

Brevinin-2 related

R. temporaria	GLWETIKNFGKKFTLNILHKLKCKIGGGC
R. septentrionalis	GIWDTIKSMGKVFAGKILQNL.NH_2
R. virgatipes a	GIWDTLKNVGKAVLGKVLENV.NH_2
R. virgatipes b	SIWDTIKNVGKTVLGKVLEIV.NH_2

Fig. 7. A comparison of the primary structures of peptides related to ranacyclin and brevinin-2 isolated from the skins of ranid frogs. The shaded residues are conserved. Residue deletions denoted by (*) have been introduced in some sequences to maximize structural similarity.

4.6. Brevinin-2-related peptides

Brevinin-2 was first isolated from the skin of the Japanese frog *P. porosus* [30] and subsequent work has shown that members of the family are widely distributed in Eurasian species but to-date brevinin-2 peptides have not been detected in any North American ranid. However, structurally-related C-terminally α-amidated peptides were isolated from skin secretions from *L. virgatipes* [42] and *L. septentrionalis* [33] that show sequence similarity to peptides belonging to the brevinin-2 family but lack the C-terminal cyclic heptapeptide domain Cys-Lys-Xaa_4-Cys, that is present in orthologs from other species (Fig. 7). Such truncated forms of brevinin-2 were not identified in the skins of other frogs belonging to the Aquarana species group. This observation provides further support for a close phylogenetic relationship between *L. virgatipes* and *L. septentrionalis* [26,28]. The relatively high potency of the peptide from *L. septentrionalis* (MIC ≤ 25 μM) against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria and against the opportunistic yeast pathogen (*C. albicans*) demonstrates that the cyclic domain in brevinin-2 is not necessary for antimicrobial activity.

Antimicrobial activity of frog skin peptides is almost always determined using reference strains or clinical isolates of microorganisms that are relevant to human disease rather than against those that the animal might encounter in the wild. However, the brevinin-2 related peptide from *L. septentrionalis* inhibited growth of isolates of the genera *Pseudomonas*, *Serratia*, *Bacillus*, *Aeromonas*, *Burkholderia*, *Microbacterium*, and *Delftia* that were obtained from the frog's natural habitat [3].

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