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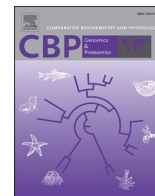
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Peptidomic analysis of the host-defense peptides in skin secretions of the Amazon River frog *Lithobates palmipes* (Ranidae)

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ABSTRACT

Skin secretions of certain frog species represent a source of host-defense peptides (HDPs) with therapeutic potential and their primary structures provide insight into taxonomic and phylogenetic relationships. Peptidomic analysis was used to characterize the HDPs in norepinephrine-stimulated skin secretions from the Amazon River frog *Lithobates palmipes* (Ranidae) collected in Trinidad. A total of ten peptides were purified and identified on the basis of amino acid similarity as belonging to the ranatuerin-2 family (ranatuerin-2PMA, -2PMb, -2PMc, and -2PMd), the brevinin-1 family (brevinin-1PMA, -1PMb, -1PMc and des(8–14)brevinin-1PMA) and the temporin family (temporin-PMA in C-terminally amidated and non-amidated forms). Deletion of the sequence VAAKVLV from brevinin-1PMA (FLPLIAGVAAKVLVLPKIFCAISKKC) in des[(8–14)brevinin-1PMA resulted in a 10-fold decrease in potency against *Staphylococcus aureus* (MIC = 31 μM compared with 3 μM) and a > 50-fold decrease in hemolytic activity but potency against *Echerichia coli* was maintained (MIC = 62.5 μM compared with 50 μM). Temporin-PMA (FLPFLGKLLSGIF.NH₂) inhibited growth of *S. aureus* (MIC = 16 μM) but the non-amidated form of the peptide lacked antimicrobial activity. Cladistic analysis based upon the primary structures of ranatuerin-2 peptides supports the division of New World frogs of the family Ranidae into the genera *Lithobates* and *Rana*. A sister-group relationship between *L. palmipes* and Warszewitsch's frog *Lithobates warszewitschii* is indicated within a clade that includes the Tarahumara frog *Lithobates tarahumarae*. The study has provided further evidence that peptidomic analysis of HDPs in frog skin secretions is a valuable approach to elucidation of the evolutionary history of species within a particular genus.

1. Introduction

Host-defense peptides (HDPs) synthesized in the skins of a range of frog species comprise a component of the animal's system of innate immunity providing protection against invasion by pathogenic microorganisms in the environment (Varga et al., 2019). In addition, they may function to facilitate the action of toxins in the secretions to provide a defense against predators (Raaymakers et al., 2017). Frog skin HDPs have excited interest as a source of potentially valuable therapeutic agents. Although their growth-inhibitory activity against bacteria and fungi, including antibiotic-resistant strains, has been the most

extensively studied (Xu and Lai, 2015; Talapko et al., 2022), the HDPs are multifunctional and other potentially important clinically relevant properties include immunomodulatory, wound healing antioxidant antiviral, anticancer, and antidiabetic actions [reviewed in (Conlon et al., 2019; Haney et al., 2019)]. However, it must be pointed out the initial promise of using the repository of biologically active peptides present in the skins of frogs as a source of new drugs has yet to be fulfilled as no compound derived from amphibian HDPs is currently in clinical practice.

Frog skin HDPs are characterized by a very high degree of variability in their primary structures and comparisons of their amino acid

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sequences can be used to complement analyses based upon morphological characteristics and comparisons of nucleotide sequences of mitochondrial genes to provide insight into the evolutionary history of species within a particular genus. Such cladistic analysis has been successfully applied to elucidation of phylogenetic relationships within the extensive families Ranidae (Conlon et al., 2009a; Mechkarska et al., 2019), Pipidae (Coquet et al., 2016) and Leptodactylidae (Barran et al., 2020).

Taxonomic analyses of the family Ranidae based upon molecular as opposed to morphological criteria have resulted in substantial revisions and reclassification of many well-known species. The former genus *Rana* Linnaeus, 1758, once regarded as a single taxon encompassing all members of the Ranidae family, is no longer regarded as constituting a monophyletic group (Frost et al., 2006; Hillis, 2007; Pyron and Wiens, 2011). At the time of writing, current recommendations divide the family Ranidae, comprising 437 species, into 27 genera with the genus *Rana* being retained for a restricted group of 58 species from Eurasia and North America. The genus *Lithobates* Fitzinger, 1843 currently comprises 51 species from North, Central and South America to southern Brazil (Frost, 2023).

The Amazon River frog *Lithobates palmipes* (Spix, 1824), also known as the Amazon Water frog, is widely distributed in northern and Amazonian South America east of the Andes with a population established in Trinidad (Murphy et al., 2018). It occupies tropical rainforests near permanent sources of water and is listed by the International Union for Conservation of Nature's Red List of Threatened Species as a species of least concern in view of its distribution and presumed large population (IUCN, 2023). However, in some countries these frogs are threatened because of the introduction of invasive frog species, habitat modifications and consumption as food.

This study has employed reversed-phase HPLC coupled with MALDI-TOF mass spectrometry and automated Edman degradation to purify and characterize the array of HDPs present in norepinephrine-stimulated secretions of *L. palmipes* frogs collected in Trinidad. The peptides are classified according to a generally accepted nomenclature recommended for designating such peptides from the Ranidae (Conlon, 2008). PM is used to denote the species of origin (*L. palmipes*) and peptides belonging to the same peptide family are differentiated by lower-case letters e.g. ranatuerin-2PMa and ranatuerin-2Pmb.

2. Materials and methods

2.1. Collection of skin secretions

All experiments with live animals were approved by the Wildlife Section, Forestry Division, Trinidad (Special Game License issued on 21/06/2016) and the University of the West Indies (UWI) Campus Ethics Committee (CEC234/07/16) and were carried out by authorized investigators. Adult *L. palmipes* ($n = 2$, sex not determined; 56 g and 67 g body weight; 84 mm and 85 mm snout-to-vent length) were collected at Saunders Trace/Trinity Inniss Field, South Trinidad (10°09'53.5"N, 61°15'42.7"W) in September 2019. The animals were sampled in the field and subsequently released unharmed at the site of capture. Each frog was injected via the dorsal lymph sac with norepinephrine hydrochloride (40 nmol/g body weight) and placed in distilled water (100 mL) for 15 min as previously described (Conlon and Sonnevend, 2010). The collection solution was acidified by addition of concentrated hydrochloric acid (0.5 mL) and immediately frozen.

2.2. Purification of the peptides

Partial purification of the pooled skin secretions on Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) and purification to near homogeneity by successive chromatographies on semipreparative (1.0 cm × 25 cm) Vydac 218TP510 (C-18), Vydac 214TP510 (C-4) and Vydac 208TP510 (C-8) reversed-phase HPLC columns (Grace, Deerfield,

IL, USA) was carried out as previously described (Barran et al., 2020). Full details are provided as Supplementary Material.

2.3. Structural characterization

MALDI-TOF mass spectrometry was carried out using an UltrafleXtreme instrument (Bruker Daltonik, Bremen, Germany). Full details of the procedure, including calibration of the instrument, have been provided previously (Conlon et al., 2018). The accuracy of mass determinations was <0.02 %. The primary structures of the purified peptides were determined by automated Edman degradation using an Applied Biosystems model 494 Procise sequenator (Applied Biosystems, Courtaboeuf, France).

2.4. Antimicrobial assays

Reference strains of microorganisms from the American Type Culture Collection (Rockville, MD, USA) were obtained from the Microbiology Research Group at the Department of Life Sciences, Faculty of Science and Technology, University of the West Indies. Minimum inhibitory concentrations (MICs) of the purified peptides against the Gram-positive bacteria ampicillin-resistant *Staphylococcus aureus* (ATCC 12600) and ampicillin- and vancomycin-resistant *Enterococcus faecium* (ATCC 19434) and against the Gram-negative bacterium *Escherichia coli* (ATCC 35218) were measured by standard microdilution methods (Clinical Laboratory and Standards Institute, 2019). Full details are provided in Conlon and Sonnevend (2010). Reproducibility and validity of the assays were monitored by parallel incubations with serial dilutions of ampicillin (*E. coli*) and vancomycin (*S. aureus*).

2.5. Hemolysis assay

Hemolytic activity of the peptides against erythrocytes from female National Institutes of Health (NIH) Swiss mice was determined as previously described (Barran et al., 2020). Full details are provided as Supplementary Material.

2.6. Cladistic analysis

The optimal phylogenetic tree based upon the amino acid sequences of 47 ranatuerin-2 peptides isolated from 21 *Lithobates* species from the New World was constructed using the neighbor-joining method. A second optimal phylogenetic tree was constructed using an expanded data set that included an additional 20 ranatuerin-2 primary structures from 7 New World species belonging to the genus *Rana*. Full details, including primary structures of the peptides used in the analyses, are provided as Supplementary Material. In both cases, the amino acid sequence of ranatuerin-2 from the European frog *Rana graeca* (Mechkarska et al., 2019) was included as out-group to polarize the in-group taxa.

3. Results

3.1. Purification of the peptides

An aliquot representing 20 % of the total amount of pooled skin secretions, after partial purification on Sep-Pak C-18 cartridges, was chromatographed on a Vydac C-18 semipreparative reversed-phase HPLC column (Fig. 1). The prominent peaks designated 1–8 were collected and subjected to further purification on semipreparative Vydac C-4 and C-8 columns. Subsequent structural analysis showed that peak 1 contained des[8–14]brevinin-1PMa, peak 2 ranatuerin-2PMa + ranatuerin-2Pmb, peak 3 ranatuerin-2PMc, peak 4 ranatuerin-2PMd, peak 5 temporin-PM non-amidated, peak 6 brevinnin-1PMa, peak 7 temporin-1PM, and peak 8 brevinnin-1Pmb + brevinnin-1PMc. The peptides were purified to near homogeneity (purity >98 % as assessed by a symmetrical peak shape and mass spectrometry) by further chromatographies

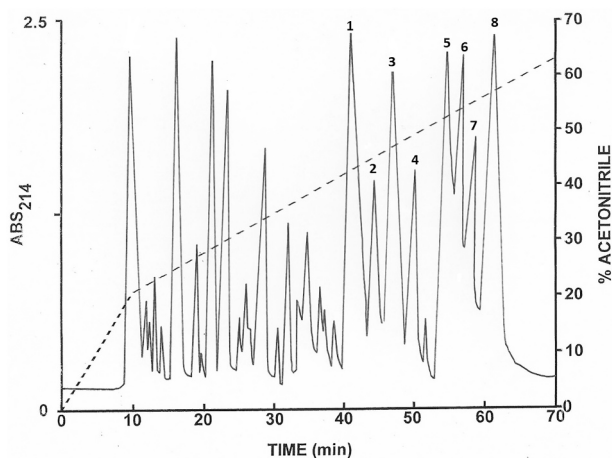


Fig. 1. Reversed-phase HPLC on a semipreparative Vydac C-18 column of pooled skin secretions from two *L. palmipes* frogs collected in Trinidad after partial purification on Sep-Pak C-18 cartridges. The dashed line shows the concentration of acetonitrile in the eluting solvent. The peaks denoted 1–8 contain host-defense peptides that were purified further.

on semipreparative Vydac C-4 and Vydac C-8 columns. The methodology is illustrated by the partial separation of brevinin-1PMB and brevinin-1PMc on a Vydac C-4 column (Fig. 2A), followed by purification to near homogeneity of brevinin-1PMB on a Vydac C-8 column (Fig. 2B). The yields of purified peptides were (nmol): des(8–14)brevinin-1PMA 39, ranatuerin-2PMA 85, ranatuerin-2PMB 16, ranatuerin-2PMc 38, ranatuerin-2PMD 27, brevinin-1PMA 76, brevinin-1PMB 47, brevinin-1PMc 86, temporin-PMa non-amidated 12, temporin-PMa 24.

3.2. Structural characterization

The amino acid sequences of the peptides isolated from *L. palmipes* skin secretions were established without ambiguity by automated Edman degradation and their complete primary structures are shown in Table 1. The molecular masses of the peptides, determined by MALDI-TOF mass spectrometry, were consistent with the proposed structures and are also shown in Table 1. The data indicate the ranatuerin-2 and brevinin-1 peptides contain an intramolecular disulfide bridge and the temporin peptides were isolated in both C-terminally α -amidated and non-amidated forms.

3.3. Antimicrobial and cytotoxicity activities

As shown in Table 2, brevinin-1PMA showed potent growth inhibitory activity against antibiotic-resistant strains of the Gram-positive bacteria *S. aureus* (MIC = 3 μ M) and *E. faecium* (MIC = 6 μ M) and was active against a reference strain of the Gram-negative bacterium *E. coli* (MIC = 50 μ M). The corresponding MIC value for ampicillin was 2.5 μ g/mL (*E. coli*) and 2.5 μ g/mL for vancomycin (*S. aureus*). The peptide was strongly hemolytic against mouse erythrocytes (LC₅₀ = 4 μ M). The antimicrobial potency of des(8–14)brevinin-1PMA containing a hepta-peptide deletion decreased 10-fold against *S. aureus* and 20-fold against *E. faecium* relative to the full-length peptide but potency against *E. coli* was not significantly different (MIC = 62.5 μ M). Hemolytic activity was markedly reduced (> 50-fold). Temporin-PMa inhibited growth of *S. aureus* (MIC = 15.6 μ M) and was strongly hemolytic (LC₅₀ = 8 μ M) but was inactive against *E. coli* (MIC >250 μ M). The non-amidated form of temporin-PMa lacked antimicrobial activity against both *S. aureus*, *E. faecium* and *E. coli* (MIC >250 μ M) and was weakly hemolytic (LC₅₀ = 110 μ M).

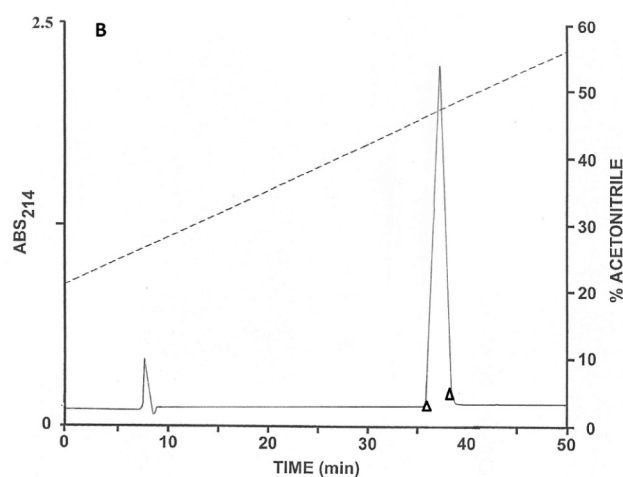
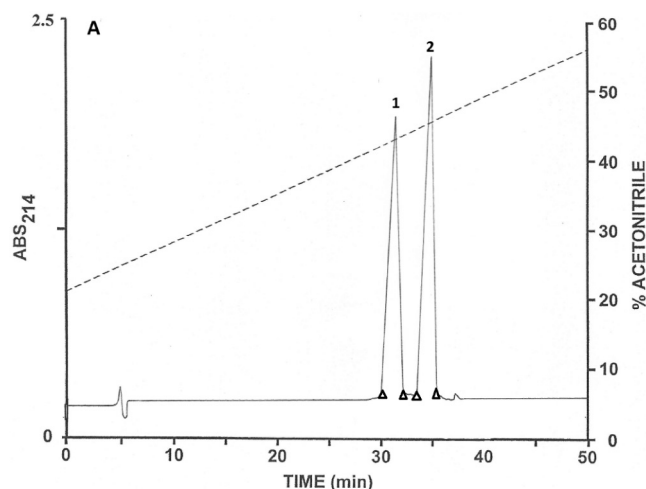


Fig. 2. (A) Separation of brevinin-1PMB (peak 1) and brevinin-1PMc (peak 2) on a semipreparative Vydac C-4 column and (B) purification to near homogeneity of brevinin-1PMB on a semipreparative Vydac C-8 column. The arrowheads show where peak collection began and ended. The dashed line shows the concentration of acetonitrile in the eluting solvent.

3.4. Cladistic analysis

The optimal phylogenetic tree based upon the amino acid sequences of 47 ranatuerin-2 peptides isolated from 21 New World species belonging to the genus *Lithobates* is shown in Fig. 3. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The optimal phylogenetic tree based upon an expanded data set that included an additional 20 ranatuerin-2 primary structures from 7 New World species belonging to the genus *Rana* is shown in Fig. 4.

4. Discussion

4.1. Characterization of host-defense peptides in *L. palmipes* skin secretions

Norepinephrine-stimulated skin secretions of *L. palmipes*, as found with other New World frogs from the genus *Lithobates* [reviewed in (Conlon et al., 2009a, Xu and Lai, 2015)], contain peptides belonging to the ranatuerin-2, brevinin-1 and temporin families in relatively high concentrations. These peptide families were first identified in their species of origin on the basis of their antibacterial and antifungal

Table 1Primary structures and molecular masses of the peptides isolated from norepinephrine stimulated skin secretions from *L. palmipes*.

Peptide	Primary Structure	$[MH^+]_{calc}$	$[MH^+]_{exp}$
Des(8–14) brevinin-1Pma	FLPLIAGKIFCAISKKC	1850.1	1850.0
Ranatuering-2Pma	GIMDSIKGAAKNLGQLLDLKLCKITGC	2887.6	2887.4
Ranatuering-2Pmb	GIMDSIKGAAKNLGELLDLKLCKITGC	2888.6	2888.4
Ranatuering-2Pmc	GIMDSIKGVAKNLGQLLDLKLCKITGC	2915.6	2915.3
Ranatuering-2Pmd	GIMDSIKGVAKNLGELLDLKLCKITGC	2916.6	2916.4
Brevinin-1Pma	FLPLIAGVAAKVLPKIFCAISKKC	2528.5	2528.5
Brevinin-1Pmb	FLPLIAGMAANFLPKIFCAISKKC	2594.4	2594.4
Brevinin-1Pmc	FLPIIAGVAAKVLPKVFCFITKCC	2604.5	2604.5
Temporin-Pma	FLPFLGKLLSGIF ^a	1450.8	1450.5
Temporin-Pma non-amidated	FLPFLGKLLSGIF	1451.8	1451.5

^a Denotes C-terminal α -amidation. $[MH^+]_{exp}$ denotes the experimentally determined molecular mass and $[MH^+]_{calc}$ denotes the mass calculated from the proposed structures.

Table 2Minimum inhibitory concentration (MIC) and hemolytic activity (LC₅₀) against mouse red blood cells (RBC) in μ M of peptides isolated from skin secretions of the Amazon River frog *Lithobates palmipes*.

Peptide	<i>E. coli</i> MIC	<i>S. aureus</i> MIC	<i>E. faecium</i> MIC	RBC LC ₅₀
Des(8–14)brevinin-1Pma	62.5	31.3	125	>200
Brevinin-1Pma	50	3.13	6.25	4
Temporin-Pma	>250	15.6	ND	8
Temporin-Pma non-amidated	>250	>250	>250	110

ND: not determined.

activities but certain members also display cytotoxicities against human tumor cells (Xiong et al., 2021), cytokine-mediated immunomodulatory properties (Popovic et al., 2012) as well as showing therapeutic potential as anti-diabetic agents by their ability to stimulate insulin release from BRIN-BD11 clonal β -cells (Mechkarska et al., 2011). The brevinin-1 and ranatuering-2 peptides are present in multiple molecular forms whose primary structures are closely related with those of ranatuering-2 paralogs being the more strongly conserved (Table 1). It is noteworthy that ranatuering-2Pma differs in structure from ranatuering-2Pmb and ranatuering-2Pmc differs from ranatuering-2Pmd only by the substitutions of Gln by Glu at a single site. It is unlikely that the Glu-containing peptides are generated artefactually by hydrolysis of the side-chain amide group of Gln during the collection and purification procedures as both peptides were obtained in high yield but it is unclear whether each peptide is a product of a separate gene or whether an enzyme analogous to mammalian glutaminase in *L. palmipes* produces deamidation in vivo.

In view of their high concentrations in the skin secretions, des(8–14) brevinin-1Pma and the non- α -amidated temporin-Pma are most likely the products of separate genes rather than post-translational modifications of brevinin-1Pma and temporin-Pma respectively. They contain structural features not usually found in members of their respective families and so it was considered worthwhile to investigate their cytotoxic properties. Structure-activity relationships of frog skin HDPs that display antimicrobial properties have highlighted the importance of molecular charge, hydrophobicity, conformation and amphipathicity in determining relative potencies against bacteria and eukaryotic cells (Chen et al., 2022). Such peptides are, with few exceptions, cationic and have the propensity to form a stable amphipathic α -helix in the environment of a phospholipid vesicle or in a solvent such that promotes peptide folding such as 50 % trifluoroethanol water (Haney et al., 2009; Han et al., 2021). NMR studies have shown that peptides of the brevinin-1 family are able to adopt a stable α -helical conformation in a membrane-mimetic environment, but the structure is associated with a stable kink at the site of the conserved Pro¹⁴ residue (Suh et al., 1999; Timmons et al., 2019). Abolition of this kink in brevinin-1Pma (formerly termed gaegurin-5) by the substitution Pro \rightarrow Ala to produce a linear

helix resulted in a decrease in antimicrobial potency (Park et al., 2002) suggesting that this structural feature is important in disrupting the integrity of the bacterial cell membrane (Kabelka and Vácha, 2021). Brevinin-1Pma is strongly cationic (net charge at pH 7 = +4) and is associated with a high degree of hydrophobicity (H = 0.762) (Fauchère et al., 1988). The hydrophobic moment μ H, a semi-quantitative measure of the degree of amphipathicity (Eisenberg et al., 1982) is 0.331. The atypical brevinin-1 peptide, (des(8–14)brevinin-1Pma (FLPLIAGKIFCAISKKC) contains the heptapeptide deletion Val⁸-Ala⁹-Ala¹⁰-Lys¹¹-Val¹²-Leu¹³-Pro¹⁴ compared with brevinin-1Pma resulting in a decrease in cationicity to +3 but the peptide maintains high hydrophobicity (H = 0.811) and amphipathicity (μ H = 0.276). Des(8–14)brevinin-1Pma displayed markedly decreased potency against *S. aureus* (10-fold) and *E. faecium* (20-fold) an even greater reduction in hemolytic activity (>50-fold) relative to the full-length peptide which may be a consequence of both the reduced cationicity and the absence of the kink-producing Pro¹⁴ residue. *E. faecium* is found in the intestinal tracts of healthy individuals but this opportunistic pathogen can cause life-threatening bacteremia, endocarditis, meningitis and infection of the urinary tract (Cattoir, 2022). In view of the fact that *E. faecium* has a high propensity to develop resistance to conventional antibiotics, des(8–14) brevinin-1Pma shows therapeutic potential as a template for development into an agent for use in infections caused by this bacterium.

The temporins are small (8–17 amino acid residues), linear peptides that have been extensively studied because of their ease of synthesis and therapeutic potential. As well as showing possibilities for development into anti-infective agents for use against antibiotic-resistant and biofilm-producing microorganisms (Mangoni et al., 2016), temporin peptides have excited interest as a result of their anti-inflammatory (Zhang et al., 2021), antiviral (Marocci et al., 2022), antiparasitic (André et al., 2020), wound healing (Di Grazia et al., 2014) and antidiabetic (Musale et al., 2018) properties. With the exception of the broad spectrum antimicrobial peptide temporin L (Bellavita et al., 2022), they display varying degrees of growth-inhibitory activity against Gram-positive bacteria and against the opportunistic yeast pathogens *Candida* spp. but are inactive against Gram-negative bacteria. However, the therapeutic potential of temporin-Pma, like that of brevinin-1Pma is limited by the fact that the peptides are strongly hemolytic. The reduction in cationicity from +2 to +1 at pH 7 in the non-amidated form of temporin-Pma compared with the α -amidated form resulted in abolition of antimicrobial activity against *S. aureus* and *E. faecium* and a substantial decrease in hemolytic activity (LC₅₀ = 110 μ M compared with 8 μ M for the α -amidated form).

4.2. Implications for phylogenetic relationships among *N. American Ranidae*

This study has provided further evidence that peptidomic analysis of HDPs in frog skin secretions is an aid to taxonomy and a useful approach to elucidation of the phylogeny of species within a particular genus

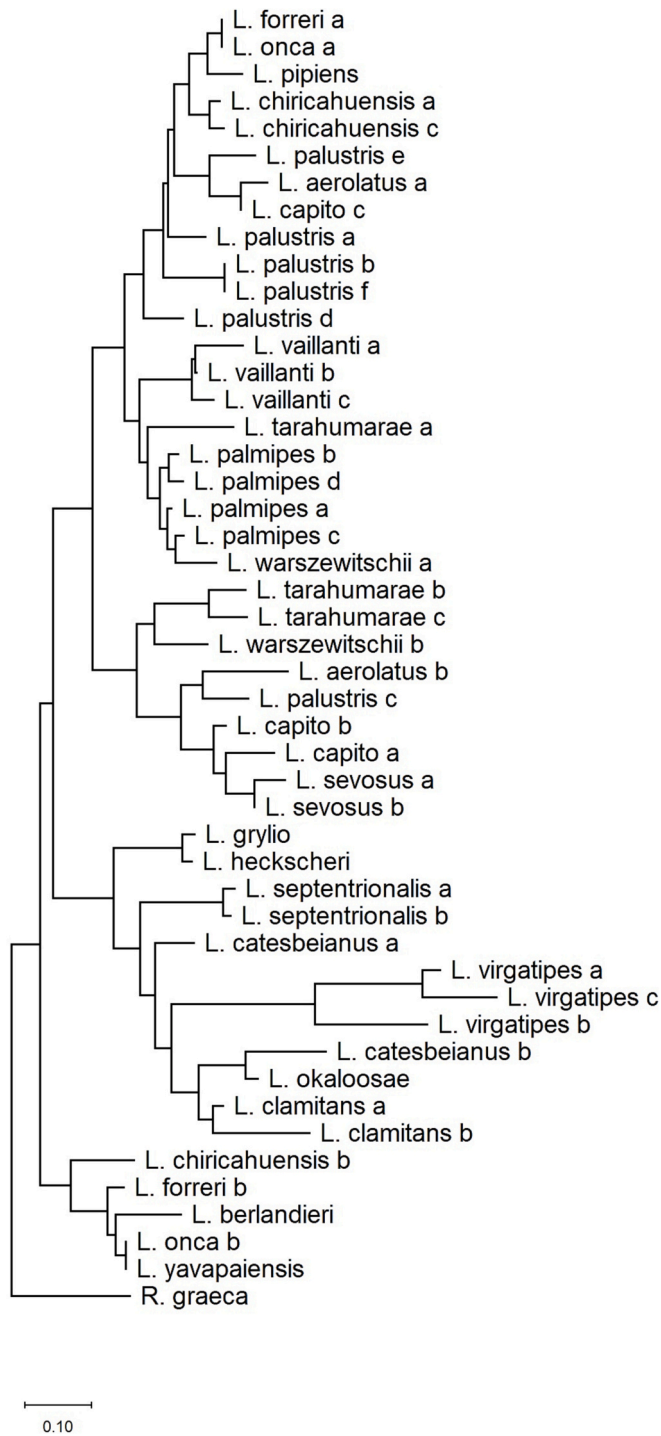


Fig. 3. A phylogenetic tree generated using the neighbor-joining method with Poisson correction based upon the primary structures of the ranatuerin-2 peptides isolated from New World frogs belonging to the genus *Lithobates*. The amino acid sequence of ranatuerin-2 from the European frog *Rana graeca* was included as out-group to polarize the in-group taxa.

(Conlon et al., 2009a; Coquet et al., 2016; Barran et al., 2020). The primary structures of ranatuerin-2 peptides have been poorly conserved between species so that the peptide represents a useful probe to investigate phylogenetic relationships among New World frogs belonging to the genera *Lithobates* and *Rana*. The present study involves an extension of a previous cladistic analysis based upon the primary structures of ranatuerin-2 peptides that used a more limited data set (Conlon et al., 2014). The data are consistent with the earlier analysis and result in an

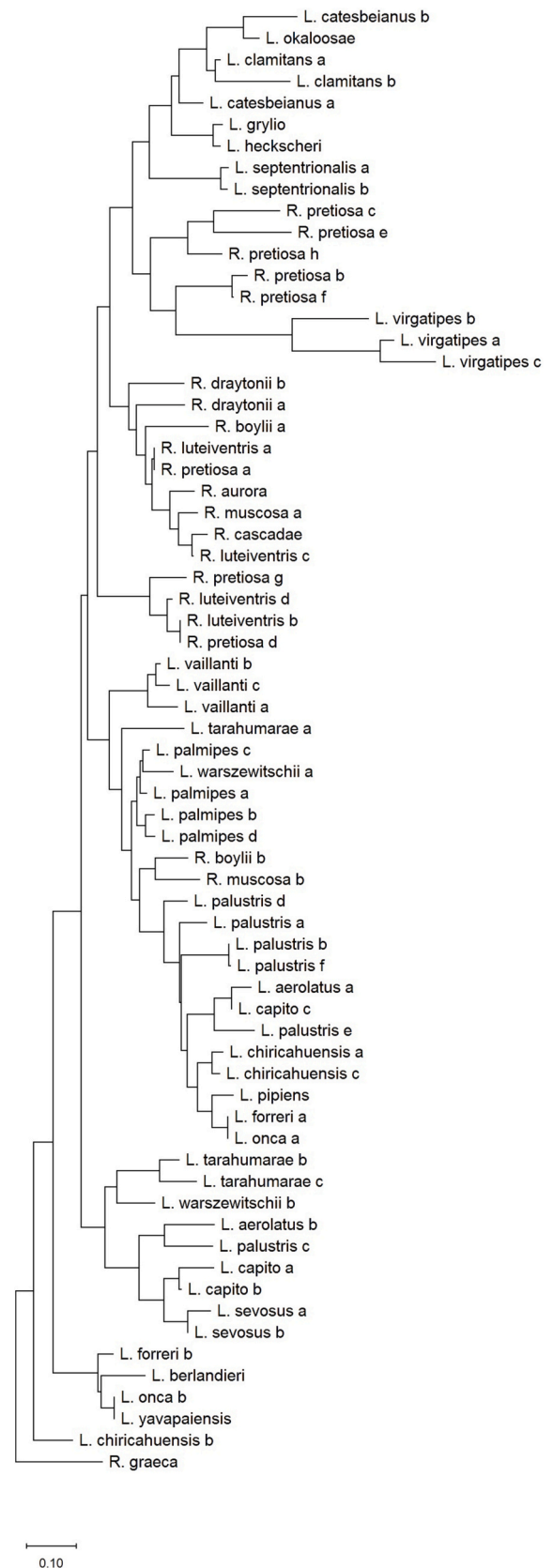


Fig. 4. A phylogenetic tree generated using the neighbor-joining method with Poisson correction based upon the primary structures of the ranatuerin-2 peptides isolated from New World frogs belonging to the genera *Lithobates* and *Rana*. The amino acid sequence of ranatuerin-2 from the European frog *Rana graeca* was included as out-group to polarize the in-group taxa.

optimal phylogenetic tree whose features generally support currently accepted evolutionary relationships based upon morphological criteria and the nucleotide sequence of paralogous genes (Fig. 3). *L. palmipes* is placed as sister-group to Warszewitsch's frog *Lithobates warszewitschii* (Conlon et al., 2009b) in a clade that also contains the Tarahumara frog *Lithobates tarahumarae* (Rollins-Smith et al., 2002). It is noteworthy that the N. American bullfrogs, formerly classified in the Aquarana species group of Hillis and Wilcox (2005), segregate in a distinct and well-defined clade. Expanding the data set to include an additional 20 ranaturin-2 primary structures from 7 New World species belonging to the genus *Rana* does not alter the proposed sister-group relationship between *L. palmipes* and *L. warszewitschii*. In addition, frogs previously classified in the Amerana species group (*Rana aurora*, *Rana boylei*, *Rana cascadae*, *Rana draytonii*, *Rana luteiventris*, *Rana muscosa* and *Rana pretiosa*) (Hillis and Wilcox, 2005) segregate together thereby providing support for a taxonomic classification in which New World frogs belonging to the Ranidae are arranged in the distinct genera *Lithobates* and *Rana* (Fig. 4).

Peptidomic analyses of skin secretions have also been used to characterize different population clusters of the same species. For example, the methodology described in the present study has provided firm evidence that the N. American red-legged frogs, *Rana aurora draytonii* and *Rana aurora aurora* should be classified as the distinct species *Rana aurora* and *Rana draytonii* (Conlon et al., 2006). Similarly the Japanese frogs previously classified *Rana tagoi okiensis* and *Rana tagoi tagoi* should be regarded as the distinct species *Rana tagoi* and *Rana okiensis* rather than sub-species (Conlon et al., 2010). In contrast, similar analysis suggested that morphologically distinct populations of the Chiricahua leopard frog *Lithobates chiricahuensis* occupying regions in southern Arizona and those from the Mogollon Rim of central Arizona are probably conspecific (Conlon et al., 2011).

It has been suggested that the population of *L. palmipes* from Venezuela may represent a distinct species for which the name *Ranula gollmeri* Peters was proposed (Hillis and Wilcox, 2005). As Trinidad is separated from the northeast coast of Venezuela by only 11 km, it will be worthwhile to compare the distribution of HDPs in skin secretions of *L. palmipes* from Trinidad determined in this study with that of frogs from the population in the coastal region of Venezuela in order to clarify their species status. If *L. palmipes* from Trinidad emerges as a distinct lineage it will represent a clear example of allopatric speciation. The indication that *L. palmipes*, *L. tarahumarae* and *L. warszewitschii* may be closely related phylogenetically was unexpected in view of the known geographical locations of the species and requires further study. *L. tarahumarae* is widely distributed in Mexico (Frost, 2023) and *L. warszewitschii* in Central America (Honduras, Panama and Costa Rica) although molecular evidence suggests that this nominal species may comprise multiple lineages (Cryer et al., 2019). It is tempting to speculate that a member of this species complex may also be present in a region closer to Trinidad such as Venezuela.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cbd.2023.101069>.

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