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Peptidomic approach identifies cruzioseptins, a new family of potent antimicrobial peptides in the splendid leaf frog, *Cruziohyla calcarifer*

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ABSTRACT

Phyllomedusine frogs are an extraordinary source of biologically active peptides. At least 8 families of antimicrobial peptides have been reported in this frog clade, the dermaseptins being the most diverse. By a peptidomic approach, integrating molecular cloning, Edman degradation sequencing and tandem mass spectrometry, a new family of antimicrobial peptides has been identified in *Cruziohyla calcarifer*. These 15 novel antimicrobial peptides of 20–32 residues in length are named cruzioseptins. They are characterized by having a unique shared N-terminal sequence GFLD– and the sequence motifs –VALGAVSK– or –GKAAL(N/G/S) (V/A)V– in the middle of the peptide. Cruzioseptins have a broad spectrum of antimicrobial activity and low haemolytic effect. The most potent cruzioseptin was CZS-1 that had a MIC of 3.77 μM against the Gram positive bacterium, *Staphylococcus aureus* and the yeast *Candida albicans*. In contrast, CZS-1 was 3–fold less potent against the Gram negative bacterium, *Escherichia coli* (MIC 15.11 μM). CZS-1 reached 100% haemolysis at 120.87 μM. Skin secretions from unexplored species such as *C. calcarifer* continue to demonstrate the enormous molecular diversity hidden in the amphibian skin. Some of these novel peptides may provide lead structures for the development of a new class of antibiotics and antifungals of therapeutic use.

Biological significance: Through the combination of molecular cloning, Edman degradation sequencing, tandem mass spectrometry and MALDI-TOF MS we have identified a new family of 15 antimicrobial peptides in the skin secretion of *Cruziohyla calcarifer*. The novel family is named "Cruzioseptins" and contains cationic amphipathic peptides of 20–32 residues. They have a broad range of antimicrobial activity that also includes effective antifungals with low haemolytic activity. Therefore, *C. calcarifer* has proven to be a rich source of novel peptides, which could become leading structures for the development of novel antibiotics and antifungals of clinical application.

1. Introduction

Antimicrobial peptides (AMPs) are a diverse group of oligopeptides that constitute the effector molecules of the innate immune response. They occur in all domains in nature, including bacteria, protozoa, fungi, molluscs, arthropods, vertebrates, and plants. AMPs have a broad spectrum of antimicrobial activity and provide protection against bacteria, fungi, parasites and viruses; however, recent research has provided evidence of additional roles in inflammation, immunity and wound healing [1].

AMPs are extremely diverse in primary structure. There is no clear correlation between structure, potency and selectivity. However, size, charge, hydrophobicity, and amphipathicity are crucial physicochemical properties for their biological activity [1,2]. Most antimicrobial peptides contain between 8 and 45 amino acids and a positive net charge of +2 to +6 at pH 7 [3]. In addition, AMPs are usually amphipathic, with a hydrophobic face containing approximately 50% of hydrophobic amino acids. The main mechanism of action involves electrostatic contact of cationic peptides with the anionic membrane of the target microorganisms followed by insertion into the membrane interior. The hydrophobic face interacts with the lipid core while the hydrophilic face interacts with the phospholipids of the cell membrane, and various models have been described, including: carpet-like, toroidal pore, and barrel-stave [1,2]. In addition, some natural AMPs undergo post translational modifications (PTMs) that are required for their antimicrobial function. Common PTMs include: phosphorylation, replacement of L-amino acids with their D-isomers, methylation, amidation, glycosylation, and disulphide bridges [4].

Amphibian skin is one of the richest sources of antimicrobial peptides. Until 2015, around 1600 AMPs had been reported from 165 species and 26 genera [5]. These peptides have been arranged into at least

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100 peptide families based on sequence similarities. Remarkably, >165 antimicrobial peptides have been reported in the dermaseptin superfamily which occurs in the skins of Central and South American frogs that belong to the Phyllomedusinae subfamily including the genera: *Phyllomedusa* (12 spp.), *Agalychnis* (5 spp.), and *Phasmahyla* (1 sp.) [5–8].

An important characteristic of the members of the dermaseptin superfamily is the highly conserved amino acid sequence in their precursor N-terminal region that correspond to the signal peptide and acidic spacer peptide. This conservation usually extends to the non-translated regions at the 5′ side of the precursor nucleotide sequence. Indeed, the extremely conserved sequences have allowed the design of primers able to target this region and have been instrumental in the discovery of a large number of related peptides. These peptides have been classified in the following families: dermaseptins sensu stricto, dermatoxins, phylloxins, phylloseptins, plasticins, medusins, caerin-related peptides and orphan peptides [8–18].

Most studies have been focused on *Phyllomedusa* and *Agalychnis*, while other genera such as *Cruziohyla* remain unexplored. *Cruziohyla* includes two species: *Cruziohyla calcarifer* that occurs in the Caribbean lowlands from eastern Honduras to the Pacific lowlands of northwestern Ecuador, and *Cruziohyla craspedopus* that occurs in the Amazon lowlands from Colombia to Peru [19]. *Cruziohyla calcarifer* was recently relocated from the genus *Agalychnis* to the new genus *Cruziohyla*[20] and, considering their taxonomic proximity to *Agalychnis*, it was presumed that this taxon also produce bioactive peptides in their skin.

Several studies have demonstrated the robustness of complementing data from shotgun molecular cloning, Edman N-terminal sequencing and tandem mass sequencing for peptidomic studies on frog skin secretions [10,21–23]. In the current study, a new family of 15 antimicrobial peptides is reported in the splendid leaf frog, *Cruziohyla calcarifer* and is named cruzioseptins. These contain an N-terminal sequence motif, GFLD– and the sequences –VALGAVSK– or –GKAAL(N/G/S) (V/A)V– in the mid-regions of their mature peptides. Cruzioseptins showed a broad spectrum of antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* with low haemolytic effects.

2. Methods

2.1. Skin secretion extraction

Two adult specimens were collected in northwestern Ecuador (Esmeraldas Province, Durango) in November 2013. Four captive reared sub-adult specimens (from Esmeraldas Province, Reserve Otokiki) were provided in 2015 by Centro Jambatu for Research and Conservation of Amphibians in Ecuador. Skin secretions were obtained after gently massaging the dorsal side of the animals. Secretions were washed off the animals with distilled water. Samples were immediately frozen and stored at −20 °C. The frogs collected in the field were returned to their habitat after the extraction. Samples were freeze-dried for analysis in Queen's University Belfast.

Twelve additional samples were taken from a group of 13-month-old captive bred frogs, whose parental line came from a Costa Rican population.Specimens were housed in terraria as pets in Belgium and Austria. Samples were extracted in the same way as described above, but instead of freeze-dried they were acidified with TFA and were transported at room temperature to the laboratory facilities in Queen's University Belfast.

2.2. Molecular cloning

Lyophilized skin secretions were dissolved in buffer A (99.95% water; 0.05% trifluoroacetic acid), pooled, and aliquoted into two tubes. One was employed for molecular cloning and the other for HPLC fractionation.

One aliquot, equivalent to skin secretion of 2.5 frogs of the Ecuadorian sample, or 1.3 mg of the Costa Rican sample, was dissolved in 1 ml of cell lysis/ binding buffer, and polyadenylated mRNA was isolated using magnetic Dynabeads Oligo (dTs) as described by the manufacturer (Dynal Biotec, UK). Isolated mRNA was subjected to 3′-rapid amplification of cDNA by using the SMART-RACE kit (Clontech, UK). In brief, three sets of 3′-RACE reactions were employed. Firstly, 3′RACE used a nested universal primer (NUP) provided with the kit and the sense primer 1 (S1: 5′-CAGCACTTTCTGAATTACAAGACCAA-3′) that was complementary to

Table 1
Antimicrobial peptides of *Cruziohyla calcarifer* identified by molecular cloning.

Peptide	Signal peptide																						Acidic spacer																						
1	*	*		*	*	*		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		*		*	*		*	*	*	*	*	*
CZS-1 (14)	M	A	F	L	K	K	S	L	F	L	V	L	F	L	G	L	V	S	L	S	I	C	E	E	E	K	R	E	E	-	N	E	E	E	Q	D	D	D	E	Q	S	E	E	K	R
CZS-2 (2)	M	A	F	L	K	K	S	L	F	L	V	L	F	L	G	L	V	S	L	S	I	C	E	E	E	K	R	E	E	E	N	E	E	V	Q	E	D	D	D	Q	S	E	E	K	R
CZS-3 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	R
CZS-4 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	R
CZS-5 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	R
CZS-6 (2)	M	A	Y	L	K	K	S	L	F	L	V	L	F	L	G	L	V	S	L	S	I	C	E	E	E	K	R	E	E	E	N	E	E	E	Q	E	D	D	D	Q	S	E	E	K	R
CZS-7 (4)	M	A	K	L	K	K	S	L	F	L	V	L	F	L	G	L	V	S	L	S	I	C	E	E	E	K	R	E	E	E	N	E	E	V	Q	E	D	D	D	Q	S	E	E	K	R
CZS-8 (6)	M	A	F	L	K	K	C	L	F	L	V	L	F	L	G	L	V	S	L	S	I	C	E	E	E	K	R	E	E	E	N	E	E	V	Q	E	D	D	D	Q	S	E	E	K	R
CZS-9 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	R
CZS-11 (6)	M	V	K	L	K	K	S	L	F	L	V	L	F	L	G	L	V	S	L	S	I	C	E	E	E	K	R	E	E	E	N	E	E	V	Q	E	D	D	D	Q	S	E	E	K	R
CZS-12(10)	M	A	F	L	K	K	S	L	F	L	V	L	F	L	G	L	V	S	L	S	I	C	E	E	E	K	R	E	E	E	N	E	E	V	Q	E	D	D	D	Q	S	E	E	K	R

	Mature peptide																														
46	*	*	*	*				*	*	*	*			*	*		*	*			*								*			
CZS-1 (14)	G	F	L	D	I	V	-	-	-	-	K	G	V	G	K	V	A	L	G	A	V	S	K	L	F	G	Q	E	E	R	*	-
CZS-2 (2)	G	F	L	D	V	I	-	-	-	-	K	H	V	G	K	A	A	L	G	V	V	T	H	L	I	N	Q	G	E	Q	*	-
CZS-3 (1)	G	F	L	D	V	V	-	-	-	-	K	H	I	G	K	A	A	L	G	A	V	T	H	L	I	N	Q	G	E	Q	*	-
CZS-4 (1)	G	F	L	D	V	I	-	-	-	-	K	H	V	G	K	A	A	L	S	V	V	S	H	L	I	N	E	G	E	H	*	-
CZS-5 (1)	G	F	L	D	V	I	K	H	V	G	K	A	V	G	K	A	A	L	N	A	V	N	D	M	V	N	K	P	E	Q	Q	S
CZS-6 (2)	G	F	L	D	V	I	T	H	V	G	K	A	V	G	K	A	A	L	N	A	V	T	E	M	V	N	Q	A	E	Q	*	-
CZS-7 (4)	G	F	L	D	V	V	K	H	V	G	K	A	V	G	K	A	A	L	N	A	V	T	E	M	V	N	Q	A	E	Q	*	-
CZS-8 (6)	G	F	L	D	V	I	K	H	V	G	K	A	A	G	K	A	A	L	N	A	V	T	E	M	V	N	Q	G	E	Q	*	-
CZS-9 (1)	G	F	L	D	V	I	T	H	V	G	K	A	V	G	K	A	A	L	N	A	V	N	E	M	V	N	Q	G	E	Q	*	-
CZS-11 (6)	G	F	L	D	I	V	K	H	V	G	K	A	A	G	K	A	A	L	N	A	V	T	E	M	V	N	Q	G	E	Q	*	
CZS-12(10)	G	F	L	D	V	V	K	H	V	G	K	A	V	G	K	A	A	L	N	A	V	N	D	L	V	N	Q	G	E	Q	*	

*Conserved sites, (x) number of clones with the same sequence. Accession numbers: KX065078-KX065088, COHK07-COHK-12.

A) Cruzioseptin-1

```
                        M  A  F     L  K  K     S  L  F  L     V  L  F
      1  TTTAGACCAA ACATGGCTTT CCTGAAGAAA TCTCTTTTCC TTGTATTATT
         L  G  L     V  S  L  S     I  C  E     E  E  K     R  E  E  N
     51  CCTTGGATTG GTCTCTCTTT CGATCTGTGA AGAAGAGAAA AGAGAAGAGA
            E  E  E     Q  D  D     D  E  Q  S     E  E  K     R  G  F
    101  ATGAAGAGGA ACAAGACGAT GATGAGCAAA GTGAAGAGAA GAGAGGCTTC
         L  D  I  V     K  G  V     G  K  V     A  L  G  A     V  S  K
    151  CTGGATATAG TAAAAGGTGT AGGAAAAGTG GCTTTAGGTG CAGTTAGTAA
         L  F  G     Q  E  E  R     *
    201  ACTTTTCGGT CAAGAAGAAC GATAAAGTTA AGAAAATGTG ATATGTCATT
    251  ACTCTAAGGA GTACAATTAT GAATAATTGT TCCAAACCTA TATAAAAAAA
    301  AAAAAAAAAA AAAAA
```

B) Cruzioseptin-2

```
                        M  A  F     L  K  K     S  L  F  L     V  L  F
      1  TTTAGACCAA ACATGGCATT CCTGAAGAAA TCCCTTTTCC TTGTACTATT
         L  G  L     V  S  L  S     I  C  E     E  E  K     R  E  E  E
     51  CCTTGGATTG GTCTCTCTTT CTATCTGTGA AGAAGAGAAA AGAGAAGAGG
            N  E  E     V  Q  E     D  D  D  Q     S  E  E     K  R  G
    101  AGAATGAGGA GGTACAAGAA GATGATGATC AAAGTGAAGA GAAGAGAGGC
         F  L  D  V     I  K  H     V  G  K     A  A  L  G     V  V  T
    151  TTCCTGGATG TAATAAAACA TGTAGGAAAA GCGGCTTTAG GTGTAGTTAC
         H  L  I     N  Q  G  E     Q  *
    201  TCACCTGATA AATCAAGGAG AACAATAAAG TCATGAAAAT GTGAAATGTC
    251  ATTACTCTAA GGAGTACAAT TATCAATAAT TGTGCCAAAC CTATATTAAA
    301  GCATATTGAA CTGACAAAAA AAAAAAAAAA AAAAAAAAAA
```

C) Cruzioseptin-3

```
         K  R  G     F  L  D  V     V  K  H     I  G  K     A  A  L  G
      1  AAGAGAGGCT TCCTGGACGT AGTAAAACAT ATAGGAAAAG CGGCTTTAGG
         ·  A  V  T     H  L  I     N  Q  G  E     Q  *
     51  TGCAGTTACT CACCTGATAA ATCAAGGAGA ACAATAAAGT CATGAAAAAG
    101  TGAAATTTCA TTACTCTGAG TACAATTATC AAAAAATGTG CCAAATCTAT
    151  ATTAAAAGAT ATTGAACAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAA
```

D) Cruzioseptin-4

```
         K  R  G     F  L  D  V     I  K  H     V  G  K     A  A  L  S·
      1  AAGAGAGGCT TCCTGGATGT AATAAAACAT GTAGGAAAAG CTGCTTTAAG
         V  V  S     H  L  I     N  E  G  E     H  *
     51  TGTAGTTTCT CATCTGATTA ATGAAGGGGA ACATTAAGGT CATGAATATG
    101  TGAAATGTCA TTACTCTAAG GAGTACTCTT ATGAGTAATT GTGCCAAACC
    151  TATATTAAAG CCTATTGTAC AGCATATTGA AAAAAAAAAA AAAAAAAAA
```

E) Cruioseptin-5

```
         K  R  G     F  L  D  V     I  K  H     V  G  K     A  V  G  K·
      1  AAGAGAGGCT TCCTGGATGT AATAAAACAT GTAGGAAAAG CTGTAGGAAA
         ·  A  A  L     N  A  V     N  D  M  V     N  K  P     E  Q  Q  S·
     51  AGCGGCTTTA AATGCAGTTA ATGATATGGT AAATAAACCA GAGCAACAAA
         ·  *
    101  GTTGAGAAAA TGTAAAACAG AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
    151  AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
```

F) Cruzioseptin-6

```
         M  A  Y     L  K  K  S     L  F  L     V  L  F     L  G  L  V·
      1  ATGGCTTACC TGAAGAAATC TCTTTTCCTT GTACTATTCC TTGGATTGGT
         ·  S  L  S     I  C  E     E  E  K  R     E  E  E     N  E  E
     51  CTCTCTTTCG ATCTGTGAAG AAGAGAAAAG AGAAGAGGAG AATGAGGAGG
         E·  Q  E  D     D  D  Q     S  E  E     K  R  G  F     L  D  V.
    101  AACAAGAAGA TGATGATCAA AGTGAAGAGA AGAGAGGCTT CCTGGATGTA
         I  T  H     V  G  K  A     V  G  K     A  A  L     N  A  V  T  ·
    151  ATAACACATG TAGGAAAAGC TGTAGGAAAA GCGGCTTTAA ATGCAGTTAC
         E  M  V     N  Q  A     E  Q  *.
    201  TGAAATGGTA AATCAAGCAG AGCAATAA
```

G) Cruzioseptin-7

```
          M  A  K     L  K  K  S     L  F  L     V  L  F     L  G  L  V·
    1     ATGGCTAAAT TGAAGAAATC TCTTTTCCTT GTGCTATTCC TTGGATTGGT
          ·  S  L  S     I  C  E     E  E  K  R     E  E  E     N  E  E  ·
   51     CTCTCTTTCG ATCTGTGAAG AAGAGAAAAG AGAAGAGGAG AATGAGGAGG
          V  Q  E  D     D  D  Q     S  E  E     K  R  G  F     L  D  V
  101     TACAAGAAGA TGATGATCAA AGTGAAGAGA AGAGAGGCTT CCTGGATGTA
          V  K  H     V  G  K  A     V  G  K     A  A  L     N  A  V  T  ·
  151     GTAAAACATG TAGGAAAAGC TGTAGGAAAA GCGGCTTTAA ATGCAGTTAC
          ·  E  M  V     N  Q  A     E  Q  *
  201     TGAAATGGTA AATCAAGCAG AGCAATAAAG TTGAGAAAAT GTAAAATCGA
  251     CAAAAAAAAA AAAAAAAAAA AAAAAAAAAA A
```

H) Cruzioseptin-8

```
          M  A  F     L  K  K  C     L  F  L     V  L  F     L  G  L  V·
    1     ATGGCTTTCC TGAAGAAATG TCTTTTCCTT GTACTATTCC TTGGATTGGT
          ·  S  L  S     I  C  E     E  E  K  R     E  E  E     N  E  E
   51     CTCTCTTTCG ATCTGTGAAG AAGAGAAAAG AGAAGAGGAG AATGAGGAGG
          V·  Q  E  D     D  D  Q     S  E  E     K  R  G  F     L  D  V
  101     TACAAGAAGA TGATGATCAA AGTGAAGAGA AGAGAGGCTT CCTGGATGTA
          I  K  H     V  G  K  A     A  G  K     A  A  L     N  A  V  T·
  151     ATAAAACATG TAGGAAAAGC TGCAGGAAAA GCGGCTTTAA ATGCAGTTAC
             E  M  V     N  Q  G     E  Q  *
  201     TGAAATGGTA AATCAAGGAG AGCAATAACG TTAAGAAAAT GTAAAATCTA
  251     ATTACTCTAA GGAGTACAAT TATCAATAAT TGTGCCAAAC CTATATTAAA
  301     GCATATTGAA CTGATAAAAA AAAAAAAAAA AAAAAAAAAA AAAA
```

I) Cruzioseptin-9

```
          K  R  G     F  L  D  V     I  T  H     V  G  K     A  V  G  K·
    1     AAGAGAGGCT TCCTGGATGT AATAACACAT GTAGGAAAAG CTGTAGGAAA
          ·  A  A  L     N  A  V     N  E  M  V     N  Q  G     E  Q  *
   51     AGCGGCTTTA AATGCAGTTA ATGAAATGGT AAATCAAGGA GAGCAATAAC
  101     GTTGAGAAAA TGTAAAATCG AATTGCGCTA AGAAGTAAAA TTATTATTAA
  151     ACTGAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAA
```

J) Cruzioseptin-11

```
          M  V  K     L  K  K  S     L  F  L     V  L  F     L  G  L  V  ·
    1     ATGGTTAAAC TGAAGAAATC TCTTTTCCTT GTATTATTCC TTGGATTGGT
          ·  S  L  S     I  C  E     E  E  K  R     E  E  E     N  E  E  V  ·
   51     CTCTCTTTCT ATCTGTGAAG AAGAGAAAAG AGAAGAGGAG AATGAGGAGG
          ·  Q  E  D     D  D  Q     S  E  E     K  R  G  F     L  D  I
  101     TACAAGAAGA TGATGATCAA AGTGAAGAGA AGAGAGGCTT CCTGGATATA
          V  K  H     V  G  K  A     A  G  K     A  A  L     N  A  V  T·
  151     GTAAAACATG TAGGAAAAGC TGCAGGAAAA GCAGCTTTAA ATGCAGTTAC
          ·  E  M  V     N  Q  G     E  Q  *
  201     TGAAATGGTA AATCAAGGAG AGCAATAAAG TTAAGAAAAT GTAAAATCTA
  251     ATTACTCTAA GGAGTACAAT TATCAATAAT TGTGCCAAAC CTATATTAAA
  301     GCATTTTGAA CAAAAAAAAA AAAAAAAAAA AAAAAAA
```

K) Cruzioseptin-12

```
          M  A  F     L  K  K  S     L  F  L     V  L  F     L  G  L  V·
    1     ATGGCTTTCC TGAAGAAATC TCTTTTCCTT GTACTATTCC TTGGATTGGT
          ·  S  L  S     I  C  E     E  E  K  R     E  E  E     N  E  E
   51     CTCTCTTTCG ATCTGTGAAG AAGAGAAAAG AGAAGAGGAG AATGAGGAGG
          V·  Q  E  D     D  D  Q     S  E  E     K  R  G  F     L  D  V
  101     TACAAGAAGA TGATGATCAA AGTGAAGAGA AGAGAGGCTT CCTGGATGTA
          V  K  H     V  G  K  A     V  G  K     A  A  L     N  A  V  N·
  151     GTAAAACATG TAGGAAAAGC TGTAGGAAAA GCGGCTTTAA ATGCAGTTAA
          ·  D  L  V     N  Q  G     E  Q  *
  201     TGATTTGGTA AATCAAGGAG AGCAATAAAG TTAAGAAGAT GTAAAATCGA
  251     ATTGCGCTAA GAAGTAAAAT TATTATTAAA CTGAGAAAAA AAAAAAAAAA
  301     AAAAAAAAAA A
```

L)

```
          ←---------1--------→  2  3 ←------4------→  5 ←-----------6-----------→  7
CZS-1     MAFLKKSLFLVLFLGLVSLSIC EEE KR EENEEEQDDDEQSEE  KR GFLDIVKGVGKVALGAVSKLF          G* QEER
CZS-2     MAFLKKSLFLVLFLGLVSLSIC EEE KR EEENEEVQEDDDQSEE KR GFLDVIKHVGKAALGVVTHLINQ        G* EQ
CZS-3                                                    KR GFLDVVKHIGKAALGAVTHLINQ       G* EQ
CZS-4                                                    KR GFLDVIKHVGKAALSVVSHLINE       G* EH
CZS-5                                                    KR GFLDVIKHVGKAVGKAALNAVNDMVNKPEQQS
CZS-6     MAYLKKSLFLVLFLGLVSLSIC EEE KR EEENEEEQEDDDQSEE KR GFLDVITHVGKAVGKAALNAVTEMVNQAEQ
CZS-7     MAKLKKSLFLVLFLGLVSLSIC EEE KR EEENEEVQEDDDQSEE KR GFLDVVKHVGKAVGKAALNAVTEMVNQAEQ
CZS-8     MAFLKKCLFLVLFLGLVSLSIC EEE KR EEENEEVQEDDDQSEE KR GFLDVIKHVGKAAGKAALNAVTEMVNQ   G* EQ
CZS-9                                                    KR GFLDVITHVGKAVGKAALNAVNEMVNQ   G* EQ
CZS-11    MVKLKKSLFLVLFLGLVSLSIC EEE KR EEENEEVQEDDDQSEE KR GFLDIVKHVGKAAGKAALNAVTEMVNQ   G* EQ
CZS-12    MAFLKKSLFLVLFLGLVSLSIC EEE KR EEENEEVQEDDDQSEE KR GFLDVVKHVGKAVGKAALNAVNDLVNQ   G* EQ
```

Table 2
Amino acid sequences of cruzioseptins confirmed by tandem mass spectrometry sequencing. Characteristic motifs of cruzioseptins are highlighted. Amidation was predicted according to the precursor sequence.

Peptide	Origin	Sequence	Identify by	Coverage %	# Peptides fragments	#AAs	LCQ MW [Da]	Theoretical average mass Da.	Score
CZS-1	ECU	GFLDIVKGVGKVALGAVSKLFa	mc, ms^2	100	110	21	2117.26	2117.60	122.74
CZS-2	ECU	GFLDVIKHVGKAALGVVTHLINQa	mc, ms^2	100	92	23	2428.40	2428.90	43.61
CZS-3	ECU	GFLDVVKHIGKAALGAVTHLINQa	mc, ms^2	100	64	23	2400.36	2400.85	10.92
CZS-4	ECU	GFLDVIKHVGKAALSVVSHLINEa	mc, ms^2	100	66	23	2445.37	2445.89	23.22
CZS-5	ECU	GFLDVIKHVGKAVGKAALNAVNDMVNKPEQQS	mc, ms^2	100	155	32	3376.79	3378.90	107.32
CZS-6	ECU	GFLDVITHVGKAVGKAALNAVTEMVNQAEQ	mc, ms^2	100	154	30	3109.62	3111.57	90.29
CZS-7	ECU	GFLDVVKHVGKAVGKAALNAVTEMVNQAEQ	mc, ms^2	100	119	30	3122.65	3124.61	70.27
CZS-8	ECU	GFLDVIKHVGKAAGKAALNAVTEMVNQa	mc, ms^2	100	117	27	2780.50	2781.28	46.11
CZS-9	ECU	GFLDVITHVGKAVGKAALNAVNEMVNQa	mc, ms^2	100	101	27	2794.48	2795.26	38.79
CZS-10	CR	GFLDVLKGVGKAALGAVTHHINNLVNQQ	Ed,ms^2	100	120	28	2912.60	2914.36	5.85
CZS-11	CR	GFLDIVKHVGKAAGKAALNAVTEMVNQa	mc, Ed,ms^2	100	104	27	2780.50	2782.26	2.91
CZS-12	CR	GFLDVVKHVGKAVGKAALNAVNDLVNQa	mc, Ed,ms^2	100	81	27	2775.54	2777.22	36.95
CZS-13	CR	GFLDVV-HVGKAVGKAALNAVNDLVNa	mc, Ed,ms^2	100	55	26	2519.39	2519.93	11.76
CZS-14	CR	GFLDIVLHVGLAAGKAALNAVNEAVNQ	Ed,ms^2	100	87	27	2703.47	2705.11	1.78
CZS-15	CR	GFLDIVKGVGLVALGAVSKS	Ed,ms^2	100	40	20	1929.13	1930.32	3.60

a= amidation, mc= molecular cloning, ms2= tandem mass spectrometry, Ed=Edman degration sequencing, accession numbers: KX065078-KX065088, COHK07-COHK-12.

the signal sequence of the phylloseptin-S5 precursor of *Phyllomedusa sauvagii*. Secondly, 3′RACE employed an NUP primer and the sense primer 2 (S2: 5′ TAGACCAAACATGGCTTTCCTGA) designed to target the signal sequence of the first antimicrobial peptide of *Cruziohyla calcarifer* (CZS-1), which was first identified with the primer sense 1 described above. The third 3'RACE included an NUP primer and the sense primer 3 (S3: 5′-AAGAGAGGCTTCCTGGAT-3′), which was also designed based on the sequence of CZS-1 but this time targeting the sequence corresponding to the first 4 amino acids of the mature sequence of the CZS-1 peptide. These primers were designed employing Primer3 and Primer-BLAST online softwares. The 3′-RACE reactions were purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated sequence.

2.3. Reverse-phase HPLC fractionation and Edman degradation

The second aliquot of freeze-dried skin secretion (corresponding to 2.5 frogs) was dissolved in 1.2 ml of buffer A (99.95% H_2O, 0.05% trifluoroacetic acid) and clarified by centrifugation. 1 ml supernatant was subjected to reverse phase HPLC employing Waters Binary pump HPLC system fitted with an analytical column Phenomenex Jupiter C18 (4.6 × 250 mm). Peptides were eluted with a linear gradient formed from 100% buffer A (99.95% H_2O, 0.05% trifluoroacetic acid) to 100% buffer B (80.00% Acetonitrile, 19.95% H_2O, 0.05% trifluoroacetic acid) in 240 min at a flow rate 1 ml/min. Fractions (1 ml) were collected every minute. Detection at 214 and 280 nm was continuous.

Skin secretion of two specimens of *C. calcarifer* from a Costa Rican population was subjected to reverse-phase HPLC using a Diphenyl column C18. Peptides were eluted in a gradient from 1% buffer A (99.95:0.05% H_2O/trifluoroacetic acid) to 80% buffer B (80.00:19.95:0.05% acetonitrile/H_2O/trifluoroacetic acid) in 80 min and fractions were collected every minute. Those fractions were tested for antimicrobial activity and the active fractions 47–53, 59 were re-chromatographed on a Vydac C18 column until clear peaks were obtained. Those samples were sequenced by automated Edman degradation. These analyses were performed in Chris Shaw lab 15 years ago (unpublished data).

2.4. MALDI-TOF MS

The molecular masses of peptides and proteins in each chromatographic fraction were analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a linear time-of-flight Voyager DE mass spectrometer (Perceptive Biosystems, MA, USA) in positive detection mode employing α-cyano-4-hydroxycinnamic acid matrix. Two microliters of sample plus 1 μl of matrix (10 mg/ml) were allowed to dry and were later analysed in the range of 500–5000 Da.

2.5. Tandem mass spectrometry sequencing

20 μl of the remaining skin secretion fraction were diluted in buffer A and was pumped directly onto an analytical HPLC column (Phenomenex C-18; 4.6 × 150 mm) connected to an LCQ Fleet ESI ion-trap mass spectrometer (Thermo Fisher, San Jose, CA, USA). The linear elution gradient was formed from 100% buffer A (99.90% H_2O, 0.1% formic acid) to 100% buffer B (19.9% H_2O, 80% acetonitrile, 0.1% formic acid) in 135 min at a flow rate 20 μl/min. Mass analysis was performed in a positive ion mode with acquired spectra in the range of m/z 500–2000 with >50% relative intensity during HPLC-MS. Parameters for electrospray ionization ion-trap mass spectrometry (ESI/MS) were: spray voltage + 4.5 kV, drying gas temperature 320 °C, drying gas flow 200 μl/min, and maximum accumulation time – for the ion trap – 350 ms. The first mass analysis was performed in full scan mode, then peptide ions with >50% relative intensity were selected for fragmentation by collision induced dissociation (CID), to generate b and y ions that were detected in a second mass analysis. The instrument was controlled by Xcalibur software (Thermo, USA) and data analysis was performed using Proteome Discover 1.0 (Thermo, USA). Sequest™ algorithm was employed to compare the acquired fragment ion profiles with the theoretical fragment ions generated from a FASTA database specific for this species built by molecular cloning (as described above) to confirm the amino acid sequences of individual peptides.

Fig. 1. Nucleotide and translated open-reading frame amino acid sequences of the sense strand of cloned cDNAs encoding cruzioseptins 1 to 9, 11 and 12 from *Cruziohyla calcarifer*. The putative signal peptides are double-underlined, acidic spacers are in italics, the mature peptides are single-underlined and the stop codons are indicated by asterisks. Accession numbers KX065078 and KX065088, respectively. A) Cruzioseptin-1 (CZS-1). B) Cruzioseptin-2 (CZS-2). C) Cruzioseptin-3 (CZS-3). D) Cruzioseptin-4 (CZS-4). E) Cruzioseptin-5 (CZS-5). F) Cruzioseptin-6 (CZS-6). G) Cruzioseptin-7 (CZS-7). H) Cruzioseptin-8 (CZS-8). I) Cruzioseptin-9 (CZS-9). J) Cruzioseptin-11 (CZS-11). K) Cruzioseptin-12 (CZS-12) L) Domain structure of the antimicrobial peptide precursors: 1. putative signal peptide. 2–5 acidic spacer peptides. 3, 5. propeptide convertase processing sites. 6. mature antimicrobial peptide. 7. C-terminal processing site with glycyl G residue amide donor indicated with an asterisk.

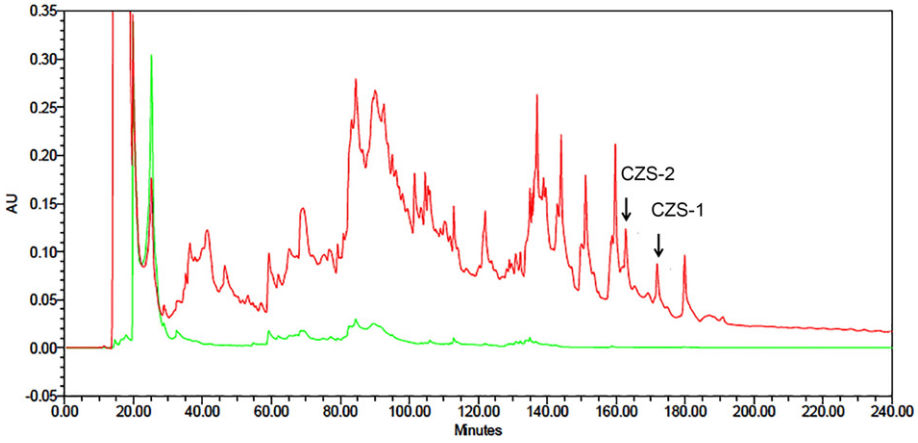

Fig. 2. Reverse phase HPLC chromatogram of *Cruziohyla calcarifer* skin secretion fractionated over 240 min with dual UV detection at 214 nm (red line) and 280 nm (green line).Arrows denote retention times of fractions with antimicrobial activity. Cruzioseptin-1 was identified in fraction 171 and Cruzioseptin-2 in fraction 162.

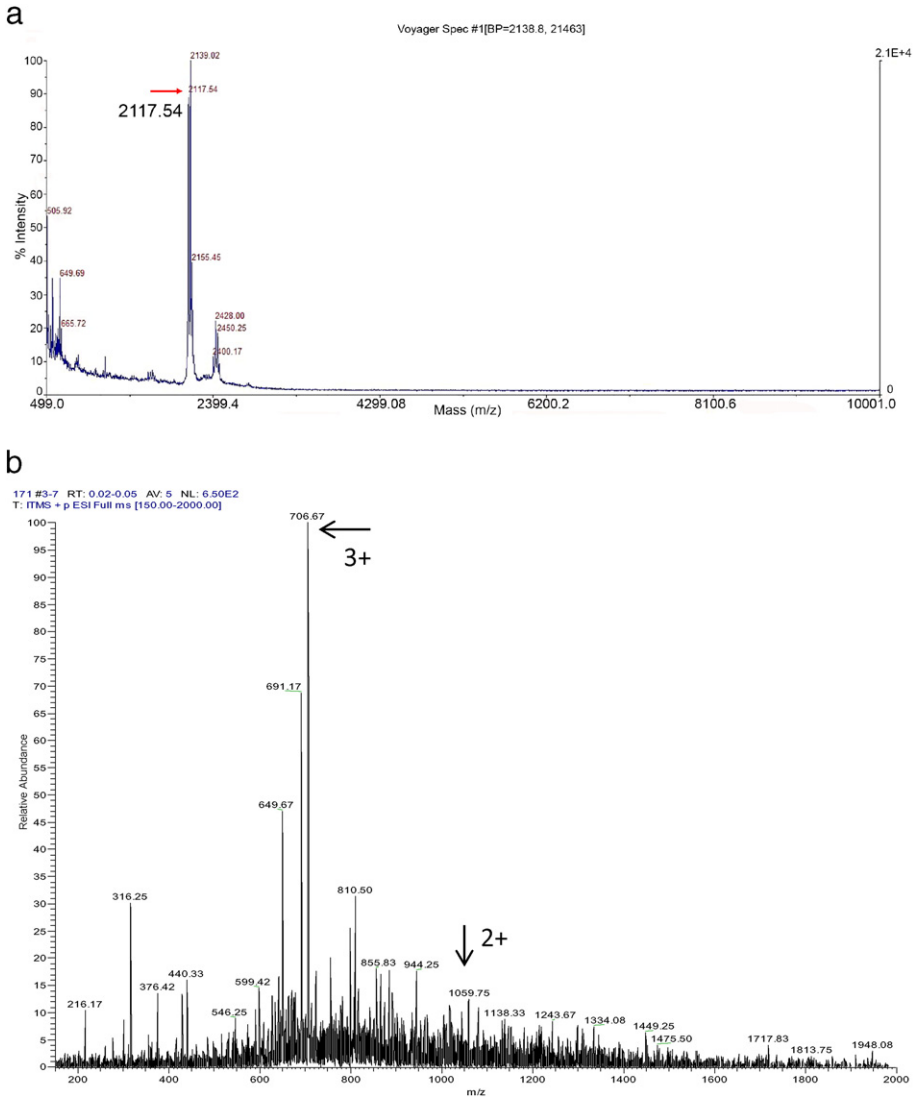

Fig. 3. Mass analysis of antimicrobial HPLC fraction with retention time 171 min containing Cruzioseptin 1. A) The arrow denotes a singly-charged ion of m/z 2117.54 obtained by MALDI-TOF MS analysis. B) LCQ MS ESI denotes precursor ions of m/z 2+ 1059.75 and 3+ 706.67 corresponding to CZS-1.

2.6. Solid phase peptide synthesis (SPPS)

Three peptides CZS-1: GFLDIVKGVGKVALGAVSKLF-amide, CZS-2: GFLDVIKHVGKAALGVVTHLINQ-amide, and CZS-3: GFLDVVKHIGKAALGAVTHLINQ-amide were chemically synthetized by solid phase Fmoc chemistry using a Tribute peptide synthesizer (Protein technologies, Inc). After cleavage from resin and de-protection, each peptide was purified by HPLC and their degrees of purity were analysed by MALDI-TOF mass spectrometry.

2.7. Antimicrobial assays

2.7.1. Antimicrobial screening

500 μl of each HPLC fraction were dried in a vacuum concentrator and later diluted in 10 μl of phosphate buffered saline (PBS). Mueller agar plates with *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* in a 10^6 CFU/ml concentration were prepared and 12 holes were prepared with a sterile Pasteur pipette. 2 μl of each fraction were transferred to one hole of each plate to be tested against the 3 microorganisms. Plates were incubated at 37 °C overnight and inhibition zones were recorded as antimicrobial activity.

2.7.2. Minimal inhibitory concentration MIC and minimal bactericidal concentration MBC assays

MICs of the synthetic peptides were determined against *E. coli*, *S. aureus* and *C. albicans*. In brief, serial dilutions of each peptide in dimethylsulphoxide (DMSO) were prepared to obtain concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2, 1×10^2 mg/L. Each microorganism in log phase was diluted to obtain the equivalent of 1×10^6 colony forming units (CFU)/ml for the bacteria and 1×10^5 CFU/ml for the yeast. Later, 2 μl of each peptide dilution were transferred to a 96 well sterile plate and 198 μl of the microorganism were added. As controls, 2 μl of DMSO was included instead of peptide and 200 μl of Mueller Hinton Broth in another well. 7 replicates per peptide concentration were performed and the experiment was repeated 3 times in order to confirm the results. Plates were incubated at 37 °C for 18–22 h. Growth was monitored at 550 nm in an ELISA plate reader. Later, 10 μl of each concentration without visual growth was sub-cultured on Mueller Hinton agar plates. Plates were incubated at 37 °C overnight. MBCs were recorded as the minimal concentration without any growth occurrence.

2.8. Haemolysis assay

A suspension of red blood cells (2%) was prepared with defibrinated horse blood (ICS Biosciences) and it was challenged with serial dilutions of the tested peptides resembling the same concentrations employed in the antimicrobial assays previously described. In brief, 200 μl of blood cell suspension were incubated with 200 μl of each diluted peptide and they were incubated at 37 °C for 120 min. Later, samples were centrifuged and supernatants were transferred to a 96 well plate. Lysis of red blood cells was analysed in an ELISA plate reader at $\lambda = 550$ nm. For negative controls, phosphate buffered saline was added to the cells instead of peptide, and for positive controls, phosphate buffered saline with 2% (v/v) Triton X-100 (Sigma-Aldrich) was employed. The concentrations that produce 100% haemolysis are reported.

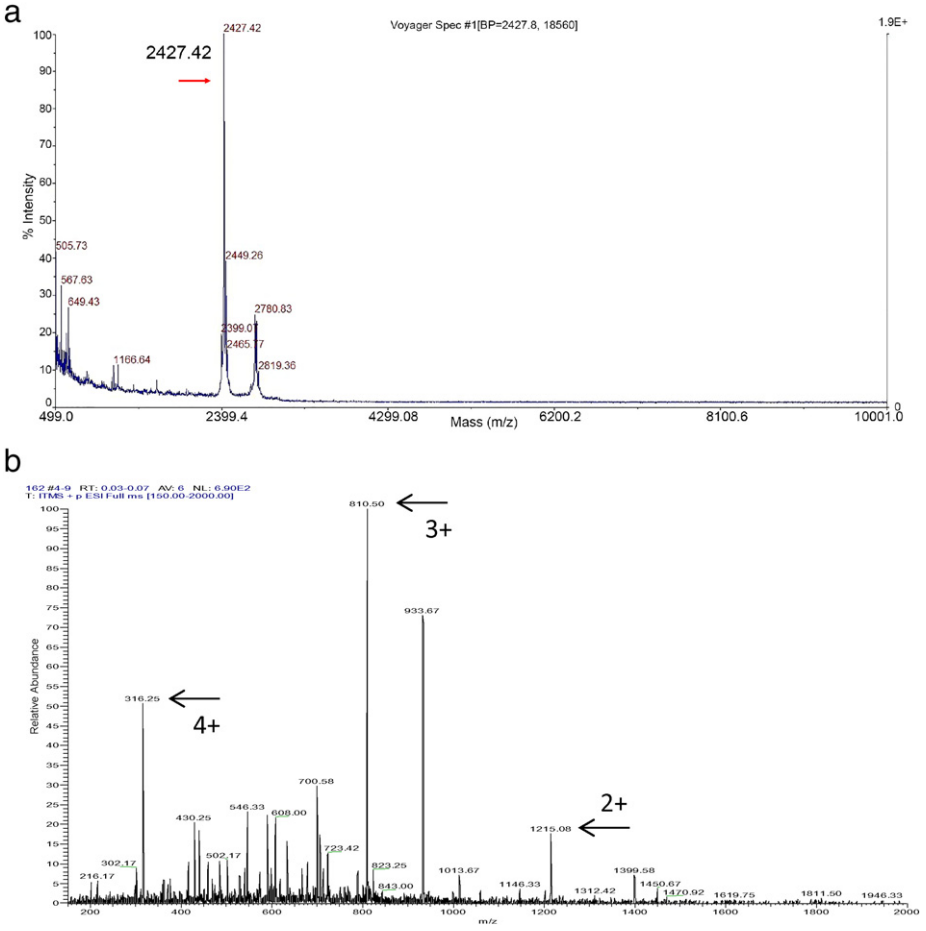

Fig. 4. Mass analysis of antimicrobial HPLC fraction with retention time 162 min containing Cruzioseptin 2. A) Arrow denotes a singly charged ion of m/z 2427.42 obtained by MALDI-TOF MS analysis B) LCQ MS ESI denotes precursor ions of m/z 2+ 1215.08, 3+ 810.50, and 4+ 316.25 corresponding to CZS-2.

2.9. Bioinformatic analysis

Nucleotide sequences were analysed by MEGA6.0 and compared by employing the BLAST tool using databases in the National Centre for Biotechnology Information (NCBI) [24,25]. Signal peptides were predicted using the SignalP 4.1 server and theoretical peptide masses were calculated with the peptide mass calculator v3.2 [26,27]. Secondary structure prediction was performed using the GOR4 programme

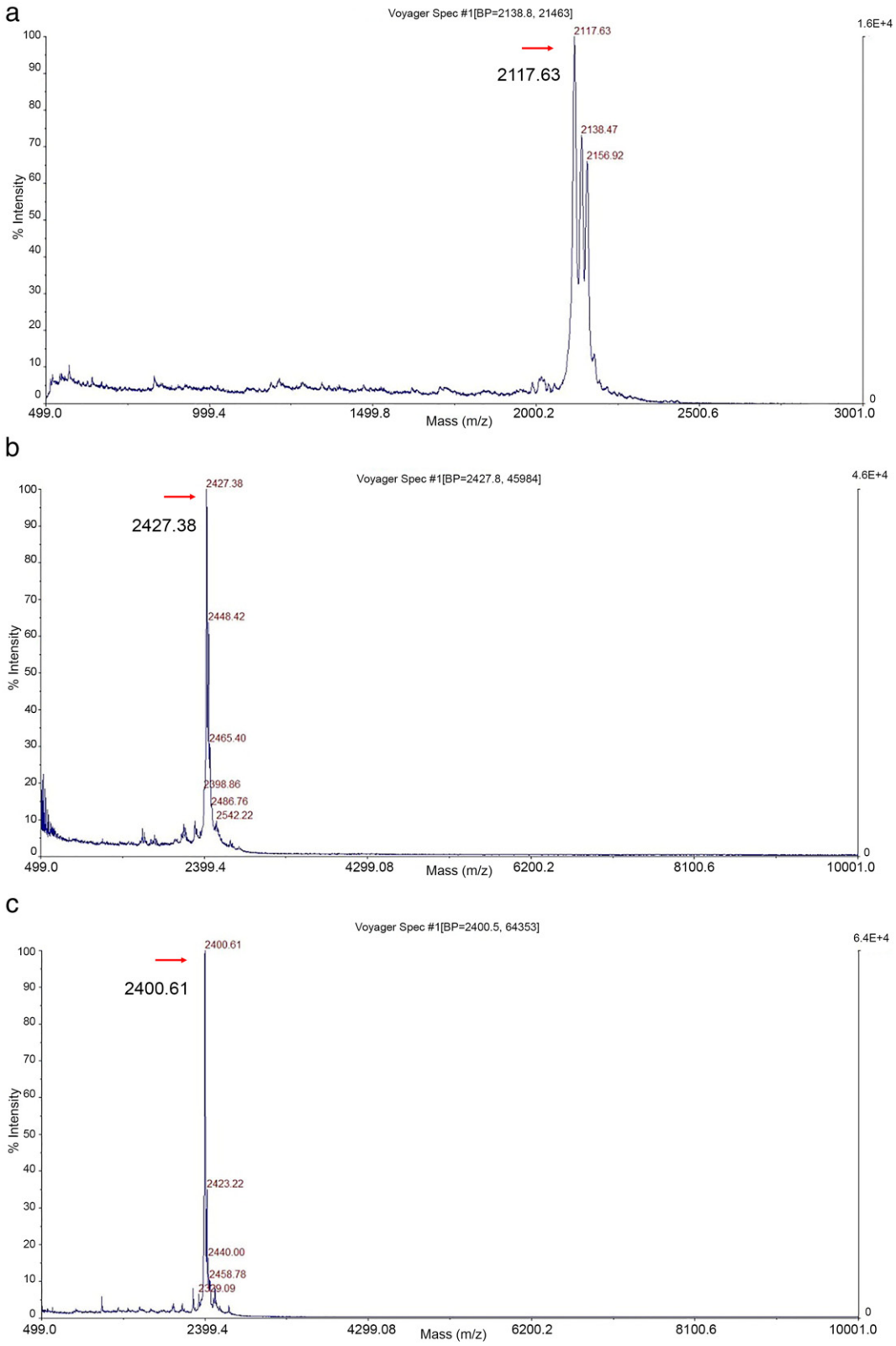

Fig. 5. Synthetic cruzioseptins 1, 2, and 3 produced by SPPS and purified by RP-HPLC. A) Cruzioseptin-1 single charge ion of m/z 2117.63. B) Cruzioseptin-2 single charged ion of m/z 2427.38. C) Cruzioseptin-3 single charged ion of m/z 2400.61.

and the physicochemical properties of the peptides were calculated using HeliQuest Computational Parameters and Peptide property calculator Bachem [28–30].

A) Cruzioseptin-1

#1	b(1+)	b(2+)	Seq.	y(1+)	y(2+)	#2
1	58.02875	29.51801	G			21
2	205.09717	103.05222	F	2060.26317	1030.63522	20
3	318.18124	159.59426	L	1913.19475	957.10101	19
4	433.20819	217.10773	D	1800.11068	900.55898	18
5	546.29226	273.64977	I	1685.08373	843.04550	17
6	645.36068	323.18398	V	1571.99966	786.50347	16
7	773.45565	387.23146	K	1472.93124	736.96926	15
8	830.47712	415.74220	G	1344.83627	672.92177	14
9	929.54554	465.27641	V	1287.81480	644.41104	13
10	986.56701	493.78714	G	1188.74638	594.87683	12
11	1114.66198	557.83463	K	1131.72491	566.36609	11
12	1213.73040	607.36884	V	1003.62994	502.31861	10
13	1284.76752	642.88740	A	904.56152	452.78440	9
14	1397.85159	699.42943	L	833.52440	417.26584	8
15	1454.87306	727.94017	G	720.44033	360.72380	7
16	1525.91018	763.45873	A	663.41886	332.21307	6
17	1624.97860	812.99294	V	592.38174	296.69451	5
18	1712.01063	856.50895	S	493.31332	247.16030	4
19	1840.10560	920.55644	K	406.28129	203.64428	3
20	1953.18967	977.09847	L	278.18632	139.59680	2
21			F-Amidated	165.10225	83.05476	1

B) Cruzioseptin-2

#1	b(1+)	b(2+)	Seq.	y(1+)	y(2+)	#2
1	58.02875	29.51801	G			23
2	205.09717	103.05222	F	2371.39736	1186.20232	22
3	318.18124	159.59426	L	2224.32894	1112.66811	21
4	433.20819	217.10773	D	2111.24487	1056.12607	20
5	532.27661	266.64194	V	1996.21792	998.61260	19
6	645.36068	323.18398	I	1897.14950	949.07839	18
7	773.45565	387.23146	K	1784.06543	892.53635	17
8	910.51456	455.76092	H	1655.97046	828.48887	16
9	1009.58298	505.29513	V	1518.91155	759.95941	15
10	1066.60445	533.80586	G	1419.84313	710.42520	14
11	1194.69942	597.85335	K	1362.82166	681.91447	13
12	1265.73654	633.37191	A	1234.72669	617.86698	12
13	1336.77366	668.89047	A	1163.68957	582.34842	11
14	1449.85773	725.43250	L	1092.65245	546.82986	10
15	1506.87920	753.94324	G	979.56838	490.28783	9
16	1605.94762	803.47745	V	922.54691	461.77709	8
17	1705.01604	853.01166	V	823.47849	412.24288	7
18	1806.06372	903.53550	T	724.41007	362.70867	6
19	1943.12263	972.06495	H	623.36239	312.18483	5
20	2056.20670	1028.60699	L	486.30348	243.65538	4
21	2169.29077	1085.14902	I	373.21941	187.11334	3
22	2283.33370	1142.17049	N	260.13534	130.57131	2
23			Q-Amidated	146.09241	73.54984	1

C) Cruzioseptin-3

#1	b(1+)	b(2+)	Seq.	y(1+)	y(2+)	#2
1	58.02875	29.51801	G			23
2	205.09717	103.05222	F	2343.36606	1172.18667	22
3	318.18124	159.59426	L	2196.29764	1098.65246	21
4	433.20819	217.10773	D	2083.21357	1042.11042	20
5	532.27661	266.64194	V	1968.18662	984.59695	19
6	631.34503	316.17615	V	1869.11820	935.06274	18
7	759.44000	380.22364	K	1770.04978	885.52853	17
8	896.49891	448.75309	H	1641.95481	821.48104	16
9	1009.58298	505.29513	I	1504.89590	752.95159	15
10	1066.60445	533.80586	G	1391.81183	696.40955	14
11	1194.69942	597.85335	K	1334.79036	667.89882	13
12	1265.73654	633.37191	A	1206.69539	603.85133	12
13	1336.77366	668.89047	A	1135.65827	568.33277	11
14	1449.85773	725.43250	L	1064.62115	532.81421	10
15	1506.87920	753.94324	G	951.53708	476.27218	9
16	1577.91632	789.46180	A	894.51561	447.76144	8
17	1676.98474	838.99601	V	823.47849	412.24288	7
18	1778.03242	889.51985	T	724.41007	362.70867	6
19	1915.09133	958.04930	H	623.36239	312.18483	5
20	2028.17540	1014.59134	L	486.30348	243.65538	4
21	2141.25947	1071.13337	I	373.21941	187.11334	3
22	2255.30240	1128.15484	N	260.13534	130.57131	2
23			Q-Amidated	146.09241	73.54984	1

Fig. 6. LCQ MS/MS Sequencing of Cruzioseptin-1 (A), Cruzioseptin-2 (B), and Cruzioseptin-3 (C). Each table contains the predicted b and y ions from each sequence. Observed ions are underlined in blue and red typefaces.

3. Results

3.1. Molecular cloning of novel antimicrobial peptide precursor-encoding cDNAs

Seven full-length and four partial-length cDNAs encoding novel peptides were cloned from the cDNA library that was constructed from the skin secretion of *Cruziohyla calcarifer* (Table 1 and Fig. 1). The novel peptides are named Cruzioseptins (CZS) to represent their origin in *Cruziohyla* –a genus in honour of a Brazilian herpetologist, Carlos Alberto Gonçalves da Cruz, in recognition of his various contributions to knowledge of Phyllomedusinae [20]. The open reading frames of these sequences contained 195–231 nucleotides. Translated amino acid sequences revealed that the precursors consisted of: (1) a putative signal peptide of 22 residues; (2) an acidic spacer peptide of 23 residues containing 2 pro-peptide convertase processing sites; and (3) a mature peptide of 20–32 residues (Fig. 1). In addition, 6 of the 15 peptides were C-terminally amidated with a Gly (G) residue as the amide donor (Table 2). Nucleotide sequences were submitted to the GenBank (NCBI) under accession numbers: KX065078–KX065088.

Each novel nucleotide sequence was analysed using the NCBI database and they showed 80–91% similarity with dermaseptins from *Phyllomedusa hypochondrialis* (Accession number AM229015.1), *Agalychnis annae* (Accession number AJ005187.1), and *P. bicolor* (Accession number Y16564.1). In addition, the BLAST/p (protein/protein) comparisons using only the translated mature sequences of these peptides, showed a lower similarity (45–90%) with dermaseptins. For example: CZS-4 was 45% similar to dermaseptin-B6 from *P. bicolor* (accession number AFR78287.1), CZS-6 was 65% similar to dermaseptin SVII from *P. sauvagii* (accession number CAD92230.1), and CZS-8 was 90% similar to dermadistinctin-L from *Phyllomedusa distincta* (accession number P83639.1). However, when the translated amino acid sequences of the mature peptides CZS-1 and 15 were subjected to BLAST/p analysis, no significant hits were found, not with any amphibian skin antimicrobial peptide or with antimicrobial peptides from other sources.

3.2. Edman degradation sequencing

Cruzioseptins 10–15 were found first by antimicrobial activity screening of reverse phase HPLC fractions of *C. calcarifer* skin secretions from the Costa Rican population. Peptides were re-chromatographed for purification and sequenced by Edman degradation. The sequences are shown in Table 2. Later, two of them were cloned from the same population, but none were cloned from the Ecuadorian population to date. The peptide sequences were submitted to the UniProt Knowledgebase under accession numbers: C0HK07- C0HK012.

3.3. Isolation and structural analysis of cruzioseptin

During functional screening of HPLC fractions of the skin secretion of *C. calcarifer*, antimicrobial activity against *S. aureus* and *C. albicans* was identified in fractions 162, 163, 171 and 172 (Fig. 2). Cruzioseptin-1 was identified in HPLC fractions 171 and 172 based on its monoisotopic molecular mass $[M + H]^{1+}$ m/z of 2117.54 as determined by MALDI-TOF mass spectrometric analysis and confirmed by LCQ ESI MS full scan that revealed ions 2+ = m/z 1059.75 and 3+ m/z = 706.67 (Fig. 3). In addition, cruzioseptin-2 was identified in HPLC fractions 162 and163 due to its monoisotopic molecular mass $[M + H]^{+1}$ m/z of 2427.42, as found by MALDI-TOF and confirmed by a LCQ ESI MS full scan, where ions 2+ m/z = 1215.08, 3+ m/z = 810.50, and 4+ m/z = 316.25, were identified (Fig. 4).

It is remarkable that all cruzioseptins 1 to 15 were 100% identified by LCQ MS/MS fragmentation sequencing employing the whole skin secretion of *C. calcarifer* (Table 2).

Table 3
Physico-chemical properties of cruzioseptins 1, 2, 3 from *Cruziohyla calcarifer*.

Peptide	Origin	Sequence/secondary structure*	Theoretical average mass Da.	Hidrophobicity <H>	Hydrophobic moment <μH>	α-helix (%)	Net charge
CZS-1	ECU	G F L D I V K G V G K V A L G A V S K L F amide c c c e e c c c c c h h h h e e e c e e c	2117.60	0.581	0.472	19.05	3.00
CZS-2	ECU	G F L D V I K H V G K A A L G V V T H L I N Q amide c c c c c e e c c c c c c c e e e e e e e e c c	2428.90	0.563	0.464	0.00	2.00
CZS-3	ECU	G F L D V V K H I G K A A L G A V T H L I N Q amide c c c c c c c c c c h h h h h h h e e e e e c	2400.85	0.523	0.441	30.43	2.00

* Secondary prediction based on GOR4: h=alpha helix, c=ramdom coil, e=extended strand, accession numbers KX065078-KX065080.

3.4. Antimicrobial and haemolytic assays of cruzioseptins

Once sequences were confirmed, cruzioseptins 1–3 were selected for further analysis. CZS 1 and 2 were chosen because these peptides were identified in HPLC fractions as detailed above, but in order to determine their potency and specificity more pure peptides were required. CZS-3 was included later due to the sequence similarity with CZS-2, aiming to determine the effect of the 3 amino acid differences in its antimicrobial activity.

Cruzioseptins 1, 2 and 3 were synthesized by solid phase Fmoc chemistry, purified by HPLC, and the sequences were confirmed by LCQ MS/MS sequencing (Figs. 5 and 6). Physico-chemical properties of CZS1–3 are summarized in Table 3. Synthetic pure peptides were employed in antimicrobial and haemolytic assays. Cruzioseptin-1 displayed potent broad-spectrum antimicrobial activity against all three microorganisms tested with MICs of 15.11 μM against *E. coli* and 3.77 μM against *S. aureus* and *C. albicans*. In addition, the MBC was below 15.11 μM for the three microorganisms. At the antimicrobial concentration of 3.77 μM, this peptide showed only 1% haemolytic activity while reaching 20% haemolysis at 15.11 μM. CZS-1 reached 100% haemolysis at 120.87 μM. In addition, cruzioseptin-2 showed moderate broad-spectrum antimicrobial activity against *E. coli* (MIC of 26.35 μM), *S. aureus* (6.59 μM), and *C. albicans* (13.18 μM). The MBC concentrations were below 52.69 μM. for the three microorganism. Nevertheless, haemolytic activity at 13.18 μM was only 26% reaching 100% haemolysis at 210.96 μM. In contrast, synthetic cruzioseptin-3 was less potent that CZS-1 and CZS-2 showing MICs of 13.32 μM against the three microorganisms tested. Moreover, the MBC was similar to CZS-2 (53.31 μM). However, haemolysis at this concentration was only 6%. CZS-3 produced 100% haemolysis at 213.33 μM. Results of these tests are summarized in Table 4 and Fig. 8.

4. Discussion

Antimicrobial peptides secreted by phyllomedusine frog skins are extremely diverse. At least eight families of antimicrobial peptides have been reported so far. These peptides have been classified based on similarities of their primary structures and/or structural motifs. The most diverse family is the dermaseptins sensu stricto, which contains >75 peptides described from 15 species [8].

Through a combination of molecular cloning, Edman degradation sequencing, and LCQ tandem MS/MS, a new family named 'cruzioseptins' of 15 antimicrobial peptides were found in the splendid leaf frog, *Cruziohyla calcarifer*. All these novel peptides share these unique structural sequences: (1) the N-terminal motif GFLD–; and (2) the motif –GKAAL(N/G/S) (V/A)V– or –VALGAVSK–. In fact, 13 of the cruzioseptins (CZS-2 to CZS-14) present the motif –GKAAL(G/N/S)(V/A)V– and 2 cruzioseptins (CZS-1 and 15) present the motif –VALGAVSK– (Table 2). Their precursor sequences are extremely conserved, sharing high similarity in the signal and acidic spacer sequences at the N-terminal ends but showing important variation in the mature sequences at their C-terminal ends. A BLAST/n search in the NCBI database identified the precursor sequences of these peptides as members of the dermaseptin superfamily. In addition, the BLAST/p comparisons with the translated mature sequences of these peptides, showed 45–90% similarity to dermaseptins. However, CZS-1 and CZS-15 did not produce any significant hits when compared with BLAST/p, suggesting that these were a well differentiated group of peptides that we recognize as a new family based on having a set of unique shared structural motifs and sequences. With a closer analysis of CZS-8, 11 and 14 sequences, it was found that the similarities with dermaseptins were concentrated in the centre of the mature peptides where these cruzioseptins share the dermaseptin motif –AAGKAALNV–. However, all cruzioseptins lack the characteristic Trp (W) in position 3 of dermaseptins. For that reason, and for having the motif GFLD– at their N-terminals, and the motifs –GKAAL(N/G/S) (V/A)V– or –VALGAVSK– at the mid-region, these novel antimicrobials were not classified as dermaseptins; instead, they were assigned to a new family of antimicrobial peptides – the cruzioseptins.

The GFLD– N-terminal motif is also found in other four amphibian skin antimicrobial peptides, including: ranatuerin-3 from *Rana catesbeiana* (accession number P82780.1), brevinins 2PTd and 2Pte

Table 4
Minimal inhibitory concentrations (MICs) and haemolytic activity of synthetic cruzioseptins from *Cruziohyla calcarifer*.

Synthetic peptide	MIC μM (mg/L)			MBC μM (mg/L)			Ha mM(mg/L)	Species	Ref.
	E. coli	*S. aureus*	*C. albicans*	*E. coli*	*S. aureus*	*C. albicans*			
CZS-1	15.11 (32)	3.77 (8)	3.77 (8)	15.11 (32)	7.56 (16)	15.11 (32)	120.87(256)	*C. calcarifer*	
CZS-2	26.35 (64)	6.59 (16)	13.18 (32)	26.35 (64)	26.35 (64)	52.69 (128)	210.96(512)	*C. calcarifer*	
CZS-3	13.32 (32)	13.32 (32)	13.32 (32)	26.66 (64)	53.31 (128)	53.31 (128)	213.33(>512)	*C. calcarifer*	
Dermaseptin-B4	5	3	NA				NA	*P. bicolor*	32
Dermadistinct-L	2.5	1.3	NA				NA	*P. distincta*	33

Ha=100% of Haemolytic activity, NA=not available.

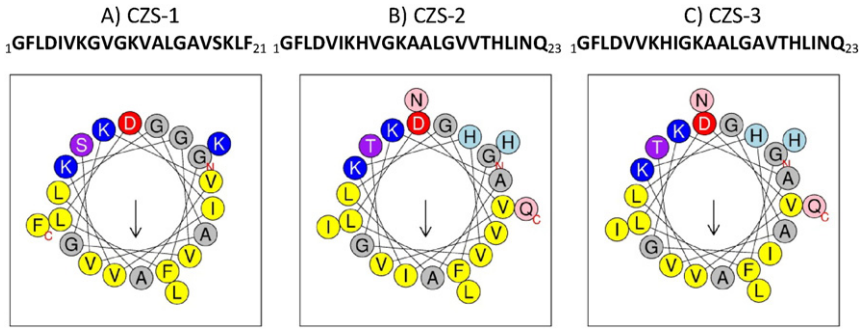

Fig. 7. Predicted alpha helical wheel plots of cruzioseptins 1, 2, and 3. Basic residues are in blue and acid residues are in red. The basic amino acid histidine is in light blue as its charge depends on pH. Non polar residues are in yellow and polar residues are in purple. Uncharged residues of glycine and alanine are in grey and asparagine and glutamine are in pink. The arrow points to the hydrophobic face.

from *Pulchrana picturata* (accession numbers POC8T6.1 and POC8T7.1, respectively), and frenatin-4 from *Litoria infrafrenata* (accession number P82023.1). These species belong to the families Ranidae and Hylidae. However, neither ranatuerin, brevinin or frenatin families contain GFLD– as a specific motif, so their appearance in these families is most likely a result of convergent evolution. On the other hand, the strongly-conserved nucleotide precursor sequences of cruzioseptins at their N-terminals in common with other members of the dermaseptin superfamily, such as litorins and caerin of the Australian frogs of the Pelodryinae subfamily, supports the view that the genetic origin of the ancestral gene precursor of cruzioseptins was present in the common ancestor which originated prior to the fragmentation of Gondwana. In addition, the extraordinary diversity of cruzioseptins found in a single species provides evidence, once again, that evolutionary mechanisms such as hypermutability of the C-terminal domain, gene duplication, and diversifying selection can provide a wide range of antimicrobial protection [9,31].

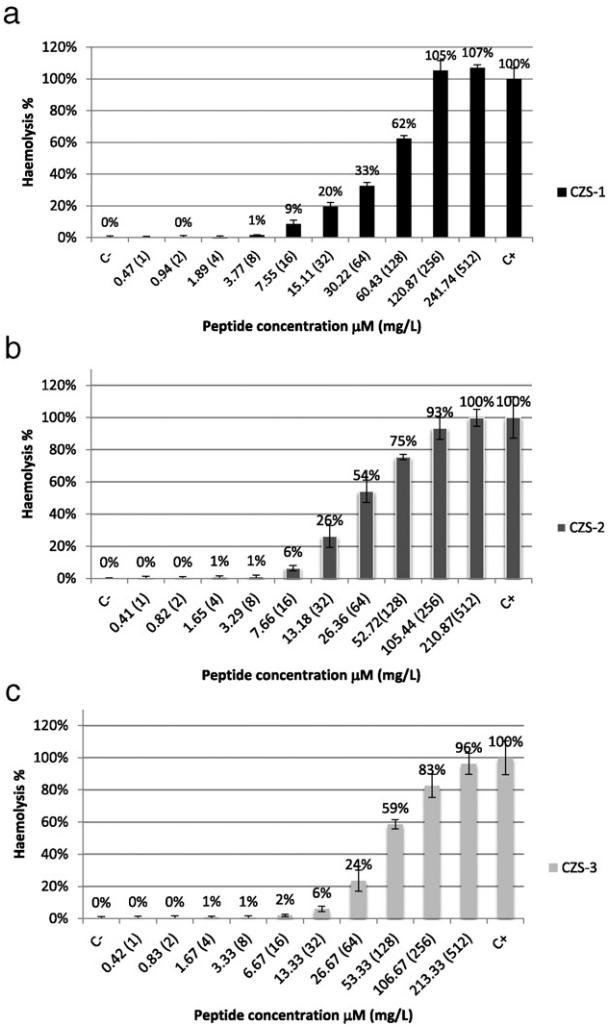

Fig. 8. Haemolytic activity of Cruzioseptins 1, 2 and 3.

In addition, three cruzioseptins were chemically synthesized and their antimicrobial profiles were analysed, showing that all three cruzioseptins (CZS1–3) have broad spectra of antimicrobial activity and relatively low haemolytic activity (Table 4). Firstly, CZS-3 showed potent activity (MIC) against the Gram negative bacterium *E. coli* at 13.32 μM, followed by CZS-1 at 15.11 μM and CZS-2 at 23.35 μM. In addition, at these concentrations, the peptides presented relatively little haemolysis (6%, 9%, and 26%, respectively) (Table 4 and Fig. 8). However, in comparison with other antimicrobial peptides of similar sequences (50–70% similarity) such as dermaseptin-B4 from *P. bicolor* (accession number P81486) and dermadistinctin-L from *P. distincta* (accession number P83639), cruzioseptins are less potent than dermaseptins, whose MICs are 5 and 2.5 μM, respectively (Table 4). Secondly, CZS-1 was the most potent of the three cruzioseptins, being able to inhibit the growth (MIC) of the Gram positive bacterium *S. aureus* at 3.77 μM; to achieve the same goal, CZS-2 is 2-fold less potent and CZS-3 is 3-fold less potent. However, dermaseptin-B4 and dermadistinctin-L are still more potent (MICs 3.0 μM and 1.3 μM, respectively) [32,33]. Finally, CZS-1 was also able to inhibit the growth (MIC) of the yeast *C. albicans* at 3.77 μM while CZS-2 and CZS-3 needed 3-fold this concentration to achieve the same goal (Table 4). Cruzioseptins 1–2 were bactericidal against *E. coli* having the same MIC and MBC concentrations. However, cruzioseptins 1–3 have a bacteriostatic effect against *S. aureus* and *C. albicans*, requiring a two or three folds concentration increase to reach the bactericidal effect (Table 4). This is an important result because there are relatively few peptides that exhibit antifungal activity and the need to develop new antifungal agents is always growing. The differences in activity found between CZS-2 and CZS-3 are very interesting because these peptides are very similar in their primary structures (87%) and both have a charge of +2. They differ only in 3 amino acids: I/V in position 6, V/I in position 9 and V/A in position 16 (Table 3).

The predicted secondary structures and physico-chemical properties of the three cruzioseptins (CZS-1, CZS-2, and CZS-3) are shown in Table 3. All three cruzioseptins have a similar hydrophobicity (H value range 0.523–0.581) and hydrophobic moment (0.441–0.472 μH), although the primary structure of CZS-1 compared to CZS-2 and CZS-3 is different sharing only 12 conserved amino acids (57%). In addition, CZS-1 has a predicted helical domain containing 19.05% of the peptide that

increases to 30.43% for CZS-3 and decreases to 0% for CZS-2. Moreover, CZS-1 possesses a higher net positive charge than CZS-2 and CZS-3 (+3 versus +2). Helical wheel plots showed that all three cruzioseptins are amphipathic having 11–13 amino acids placed in the hydrophobic face (V/I/A/F/L/G) and 8–12 amino acids hydrophilic residues placed at the opposite side (Table 3 and Fig. 7).

These 3 variations in sequence change the potency of CZS-2 making it weaker that CZS-3 against *E. coli* (13.33 vs 26.35 μM) but more potent against *C. albicans* (13.33 vs 6.59 μM).

In summary, the antimicrobial potency observed for CZS-1 could be due to its +3 charge, in contrast to the +2 of CZS-2 and CZS-3. However, in comparison with other antimicrobial peptides such as dermaseptin-B4 and dermadistinctin-L, CZS-2 is weaker against *E. coli*, but potent against *S. aureus*. Moreover, CZS showed potency against *S. aureus* and *C. albicans* with only 1% haemolysis at those concentrations, which makes CZS-1 an interesting peptide and warrants further study into its potential antibiotic and antifungal functions.

In conclusion, cruzioseptins, a novel antimicrobial peptide family, is reported in *Cruziohyla calcarifer*. Three synthetic cruzioseptins displayed broad-spectrum antimicrobial activity against *S. aureus*, *C. albicans* and less potently against *E. coli* with minor haemolytic activity. These data show once again, the phenomenal peptide diversity produced in the skin of phyllomedusine frogs such as the previously unstudied *C. calcarifer*. Interplay between molecular cloning and tandem mass spectrometry sequencing, together with functional studies of natural and synthetic peptides have proven to be a robust, cost-effective strategy for peptidomic analysis in species where databases are not available. In addition, these techniques are sensitive enough to generate data with only a few milligrams of material, and this is especially beneficial in the analysis of endangered species where samples are limited. Finally, the discovery of novel natural antimicrobial peptides such as cruzioseptins is a key element in the development of new therapeutic drugs based on the structures of natural compounds.

Conflictofinterest

The authors declare that there is no conflict of interest.

Authorship

This study was conceived and designed by CS, MZ, TC. Sample collections were performed by CPB and LAC. Data were acquired by CPB. LC-MS/MS analysis was performed by LW. The article was written by CPB and reviewed critically by CS and LAC.

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