Peptides 63 (2015) 118–125

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Evidence from peptidomic analysis of skin secretions that allopatric populations of *Xenopus gilli* (Anura:Pipidae) constitute distinct lineages

J. Michael Conlon[a,b,*], Milena Mechkarska[a], Laurent Coquet[c,d], Jérôme Leprince[e], Thierry Jouenne[c,d], Hubert Vaudry[e], G. John Measey[f]

[a] Department of Biochemistry, College of Medicine and Health Sciences, United Arab Emirates University, 17666 Al Ain, United Arab Emirates
[b] SAAD Centre for Pharmacy and Diabetes, School of Biomedical Sciences, University of Ulster, Coleraine BT52 1SA, UK
[c] PISSARO, Institute for Research and Innovation in Biomedicine (IRIB), University of Rouen, 76821 Mont-Saint-Aignan, France
[d] CNRS UMR 6270, University of Rouen, 76821 Mont-Saint-Aignan, France
[e] INSERM U-982, PRIMACEN, CNRS, IRIB, University of Rouen, 76821 Mont-Saint-Aignan, France
[f] Centre for Invasion Biology, Department of Botany & Zoology, Stellenbosch University, Stellenbosch, South Africa

ARTICLE INFO

Article history:
Received 10 October 2014
Received in revised form
18 November 2014
Accepted 18 November 2014
Available online 26 November 2014

Keywords:
Antimicrobial peptide
Frog skin
Allopatric
Pipidae, *Xenopus*
Magainin

ABSTRACT

The International Union for Conservation of Nature (IUCN) Endangered Cape Platanna *Xenopus gilli* inhabits disjunct ranges at the tip of Cape Peninsula and near the town of Kleinmond on opposite sides of False Bay in the extreme southwest of Africa. Peptidomic analysis of host-defense peptides in norepinephrine-stimulated skin secretions from frogs from the Cape Peninsula range resulted in the identification of two magainins, two peptide glycine–leucine–amide (PGLa) peptides, two xenopsin-precursor fragment (XPF) peptides, nine caerulein-precursor fragment (CPF) peptides, and a peptide related to peptide glycine–glutamine (PGQ) previously found in an extract of *Xenopus laevis* stomach. The primary structures of the peptides indicate a close phylogenetic relationship between *X. gilli* and *X. laevis* but only magainin-1, PGLa and one CPF peptide are identical in both species. Consistent with previous data, the CPF peptides show the greatest antimicrobial potency but are hemolytic. There are appreciable differences in the expression of host-defense peptide genes in frogs from the population of animals sampled near Kleinmond as peptides corresponding to magainin-G2, XPF-G1, XPF-G2, and four CPF peptides, present in secretions from the Cape Peninsula frogs, were not identified in the skin secretions from Kleinmond frogs. Conversely, PGLa-G3, XPF-G3, and three CPF peptides were identified in the Kleinmond frogs but not in the Cape Peninsula animals. The data support the conclusion from morphometric analyses and comparisons of the nucleotide sequences of mitochondrial genes that the disjunct populations of *X. gilli* have undergone appreciable genetic, morphological, and phenotypic divergence.

Introduction

Skin secretions from several species of Anura (frogs and toads) contain peptides, often in very high concentrations, with cytotoxic activities against prokaryotic and eukaryotic cells [3]. These peptides are usually identified on the basis of their ability to inhibit the growth of bacteria and so are most commonly referred to as antimicrobial peptides. However, more recent work has shown that these compounds are multifunctional, displaying anti-cancer, anti-viral, chemoattractive, and insulin-releasing properties as well as cytokine-mediated immunomodulatory activities (reviewed in [6]). Consequently, it is more informative to refer to them by the more general term host-defense peptides.

Skin secretions of clawed frogs belonging to the family Pipidae have proved to be a particularly rich source of host-defense peptides [4]. The Pipidae currently comprise 33 well-characterized species distributed in five genera: *Hymenochirus*, *Pipa*, *Pseudhymenochirus*, *Silurana*, and *Xenopus* [15]. All are found in Africa, south of the Sahara, except for members of the genus *Pipa* which are found in South America. The widely distributed common clawed frog *Xenopus laevis* was among the first amphibian species in which host-defense peptides with cytotoxic activities were

* Corresponding author at: SAAD Centre for Pharmacy and Diabetes, School of Biomedical Sciences, University of Ulster, Coleraine BT52 1SA, UK.
Tel.: +44 28 70832917; fax: +44 28 70124965.
E-mail address: m.conlon@ulster.ac.uk (J.M. Conlon).

http://dx.doi.org/10.1016/j.peptides.2014.11.005
0196-9781/

identified [16,17,40]. Analysis of *X. laevis* skin secretions has led to the isolation and characterization of magainin-1 and -2, peptides that are encoded by the same gene [40], peptide glycine–leucine-amide (PGLa), xenopsin-precursor fragment (XPF) peptides derived from the post-translational processing of the biosynthetic precursors of xenopsin, and multiple caerulein-precursor fragment (CPF) peptides derived from the post-translational processing of the biosynthetic precursors of caerulein [16]. Although these peptides do not contain conserved amino acid sequences that determine biological activity, they are cationic (a charge of between +2 and +7 at pH 7), contain between 40 and 70% hydrophobic amino acids, and have the propensity to adopt an amphipathic α-helical conformation over at least part of their structures in a membrane-mimetic solvent such as 50% trifluoroethanol–water or in the presence of a phospholipid vesicle [3,39].

More recently, host-defense peptides belonging to these families have been isolated from norepinephrine-stimulated skin secretions from other species within the *Xenopus* genus, notably the tetraploid frogs *Xenopus borealis*, *Xenopus clivii*, *Xenopus fraseri*, *Xenopus muelleri*, *Xenopus petersii*, *Xenopus pygmaeus*, and *Xenopus victorianus* and the octoploid frogs *Xenopus amieti*, *Xenopus andrei*, *Xenopus lenduensis*, *Xenopus vestitus*, and *Xenopus wittei* (reviewed in [4,5]). Host-defense peptides have also been isolated from laboratory-generated F1 hybrids of *X. laevis* × *X. muelleri* [29] and *X. laevis* × *X. borealis* [30] and from representatives of the genera *Silurana* [7], *Hymenochirus* [23,31] and *Pseudhymenochirus* [8]. Frogs from the genus *Pipa* do not appear to produce the cationic, α-helical peptides found in skin secretion of species from the other Pipidae genera [3,22].

In common with other clawed frogs, the Cape Platanna *X. gilli* Rose and Hewitt, 1927 is predominantly aquatic and inhabits undisturbed lowland blackwater pools characteristic of the fynbos biome. The species is strongly acidophilic and larvae are particularly intolerant of alkaline substrates [34]. Although believed to be once more widely distributed throughout the Cape Flats region in the southwest region of South Africa, *X. gilli* is now confined in a few scattered ponds in three disjunct ranges [14,35]. These are located at the tip of the Cape Peninsula and at two sites to the east at opposite side of False Bay near the towns of Kleinmond and Pearly Beach. *X. gilli* is classified as Endangered by the International Union for Conservation of Nature (IUCN) Red List [37]. The species does not tolerate of alteration of habitat and loss of range due to urbanization, pollution from agricultural run-off, and invasion by *X. laevis* and alien plants have contributed to population declines.

Even though the Cape Peninsula and Kleinmond ranges are less than 100 km apart, there is evidence from morphometric and genotyping data that the two populations of *X. gilli* have undergone appreciable divergence since their time of separation. Animals from the Cape Peninsula range have significantly broader and higher heads and shorter limbs than those from the Kleinmond population and differences in color and lateral line patterning on their ventral surfaces are apparent [14]. Comparisons of nucleotide sequences of mitochondrial genes have demonstrated a 3.6% patristic divergence between the two groups that is fixed in each population [12]. The magnitude of this divergence is comparable to that seen in the same region of mitochondrial DNA between sister-group species within the genus, for example *X. vestitus* and *X. lenduensis* [10]. A later study involving two molecular markers (ND2 and 16S) provided an estimated divergence time between the Cape Peninsula and eastern populations within the Pliocene (4.6 Mya with a 95% confidence interval from 3.2 to 6.4 Mya) [14]. In contrast, there was no indication from morphometric or molecular analyses that the populations from Kleinmond and Pearly Beach, on the same side of False Bay, represented separate populations [14].

The aim of the present study was to use peptidomic analysis (reversed-phase HPLC coupled with MALDI-TOF mass spectrometry) to identify and characterize the host-defense peptides in norepinephrine-stimulated skin secretions of *X. gilli* inhabiting the Cape Peninsula and the Kleinmond ranges. There is no universally accepted nomenclature for designating antimicrobial peptides from frogs of the Pipidae family and so the peptides described in this study are classified according to the terminology used previously for peptides from *X. laevis* [16]. The magainin, PGLa, CPF, and XPF peptide families are recognized. The species origin is denoted by G for *gilli*. Peptides from paralogous genes are differentiated by numerals e.g. magainin-G1 and magainin-G2.

Experimental

Collection of skin secretions

A permit to collect *X. gilli* specimens in the Western Cape Province was issued by CapeNature (permit No. AAA007-00092-0056). All experiments with live animals were approved by the Animal Research Ethics committee of U.A.E. University (Protocol No. A21-09) and were carried out by authorized investigators. Adult and sub-adult *X. gilli* from the Cape Peninsula range ($n = 8$; all female; weight range 8–18 g) were collected at three ponds in the Cape of Good Hope Nature Reserve within the Table Mountain National Park in October 2013. These ponds are monitored on a continuous basis by members of the National Park staff and any *X. laevis* are removed. The *X. gilli* individuals could be clearly differentiated from *X. laevis* on the basis of morphology, and their identity was confirmed by taking the second phalange of the right hind foot of each individual, as previously described [14], for subsequent genotyping. Adult and sub-adult *X. gilli* from the Kleinmond range ($n = 10$; 8 female; weight range 8–18 g) were collected at Lamloch Pond, Kleinmond in October 2013. This pond contained both *X. gilli*, and *X. laevis*, and probably *X. gilli* × *X. laevis* hybrids that cannot be unambiguously identified by morphological criteria. Consequently, the second phalange of the right hind foot of each individual was also clipped for subsequent genotyping.

Each animal was injected via the dorsal lymph sac with norepinephrine hydrochloride (40 nmol/g body weight) and placed in a solution (50 mL) of distilled water for 15 min. The frog was removed and the collection solution was acidified by addition of concentrated hydrochloric acid (0.5 mL) and immediately frozen for transport to U.A.E. University. All animals were released unharmed at the exact site of collection within 24 h. The solutions containing the secretions from the Cape Peninsula group were pooled and passed at a flow rate of 2 mL/min through 8 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series. Bound material was eluted with acetonitrile/water/trifluroacetic acid, TFA (70.0:29.9:0.1, v/v/v) and freeze-dried. Full details of the procedure have been provided [2]. The secretions from one female and one male frog from the Kleinmond range were separately passed at a flow rate of 2 mL/min through 6 Sep-Pak C-18 cartridges connected in series. Bound material was eluted with acetonitrile/water/TFA (70.0:29.9:0.1, v/v/v) and freeze-dried.

X. gilli genotyping

As recent studies have documented occurrence of *X. laevis* × *X. gilli* hybrids at both collection sites [14], it was necessary to sequence both mitochondrial (mt) and nuclear (n) DNA markers on all animals sampled for this study to confirm that no cryptic hybrids were present. In addition to the animals in this study, a tissue sample from a known hybrid and a *X. laevis* individual were used to validate the technique. Total genomic DNA was extracted from toe-clips using a standard salt extraction, and mtDNA sequences of ND2 were produced using the exact same protocols detailed

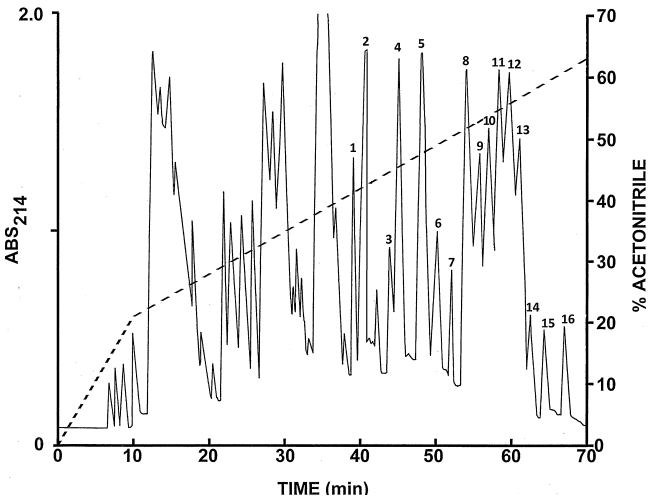

Fig. 1. Reversed-phase HPLC on a preparative Vydac C-18 column of skin secretions from *X. gilli* from the Cape Peninsula range after partial purification on Sep-Pak cartridges. The peaks designated 1–16 contained host-defense peptides and were purified further. The dashed line shows the concentration of acetonitrile in the eluting solvent.

elsewhere [14]. In addition, two nDNA markers were sequenced: recombination activation gene 2 (RAG-2) and androgen receptor hypervariable region (ARHR) [9]. The following primer sets were used for ARHR (forward 5′ ATGGCGGTGCA CATAGGGCT 3′; reverse 5′ CGGGGGTCTCTTCGCTCTCCA 3′) and RAG-2 (forward 5′ ACCTACACAGTTGCTGTGATG 3′; reverse 5′ CCCATATCAG CACCAAACC 3′). 25 μL reaction mixtures were amplified with standard PCR conditions. Electrophoresis was used to examine the quality of PCR products on a 2% agarose gel stained with Goldview and the high quality products were analyzed on an ABI Prism 3100 or 3130 XL 16-capillary genetic analyzer (Applied Biosystems). Sequences of each gene were aligned using Geneious Pro (v. 5.0.4), and the alignment was used to determine the genotype of the individuals sampled.

Peptide purification

The freeze-dried skin secretions from the Cape Peninsula group of frogs, after partial purification on Sep-Pak cartridges, were redissolved in 0.1% (v/v) TFA/water (4 mL) and injected onto a (2.2 cm × 25 cm) Vydac 218TP1022 (C-18) reversed-phase HPLC column (Grace, Deerfield, IL, USA) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 6.0 mL/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 63% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 nm and fractions (1 min) were collected using a Gilson 201 fraction collector. Previous studies with skin secretions from *Xenopus* species have shown that, under the standardized conditions of chromatography shown in Fig. 1, the relatively hydrophobic host-defense peptides are eluted from a preparative C-18 column at concentrations of acetonitrile >38%. Consequently, all major peaks with retention times >34 min were analyzed by MALDI-TOF mass spectrometry. Peptides with molecular masses in the range 1500–3000, which encompasses the mass range of the host-defense peptides previously isolated from other *Xenopus* species, were identified and subjected to further purification. Peptides in this mass range were successively chromatographed on a (1.0 cm × 25 cm) Vydac 214TP510 (C-4) column and a (1.0 cm × 25 cm) Vydac 208TP510 (C-8) column. The concentration of acetonitrile in the eluting solvent was raised from 21% to 56% over 50 min and the flow rate was 2.0 mL/min.

Skin secretions from the female and male frogs from the Kleinmond range, after partial purification on Sep-Pak cartridges, were separately chromatographed on the preparative Vydac 218TP1022 (C-18) reversed-phase HPLC column under the same conditions used for secretions from the Cape Peninsula frogs. Host-defense peptides from the female frog were purified to near homogeneity on semipreparative Vydac C-4 and C-8 columns as previously described.

Structural characterization

The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 494 Procise sequenator (Foster City, CA, USA). MALDI-TOF mass spectrometry was carried out using a Voyager DE-PRO instrument (Applied Biosystems) that was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 20 kV. The instrument was calibrated with peptides of known molecular mass in the 2–4 kDa range. The accuracy of mass determinations was ±0.02%.

Antimicrobial and hemolytic activities

Reference strains of microorganisms were purchased from the American Type Culture Collection (Rockville, MD, USA). Minimum inhibitory concentrations (MIC) of the purified peptides against reference strains of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25726) were measured in the concentration range of 3–80 μM by a standard microdilution method [1] and were taken as the lowest concentration of peptide where no visible growth was observed. The values were confirmed by measurement of absorbance at 630 nm using a microtiter plate reader. In order to monitor the validity and reproducibility of the assays, incubations were carried out in parallel with increasing concentrations of ampicillin as previously described [6].

Hemolytic activity against human erythrocytes taken from a healthy donor was measured as previously described [7]. The LC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis in two incubations carried out in duplicate.

Results

X. gilli genotyping

Sequences of the mtDNA gene ND2 were obtained for all *X. gilli* sampled as well as the additional *X. laevis* and known hybrid individual. These gave 900 bp sequences that directly aligned to haplotypes in previous studies [14]. No mtDNA sequences of *X. laevis* were found in any of the animals with morphology of *X. gilli* from either site sampled. However, the sequences from both the hybrid and *X. laevis* were found closely matched (>98% alignment) to the *X. laevis* ND2 region of Genbank accession HM991335. The nDNA sequences provided 335 bp and 900 bp for ARHR and RAG-2, respectively. The known hybrid showed intermediate base pairs at all sites where *X. laevis* differed from *X. gilli*. However, no *X. gilli* animals sampled from either site showed this pattern of hybridization. The combined results from both mtDNA and nDNA lead to the conclusion that none of the animals sampled from either population were hybrids, and that all could be considered pure bred *X. gilli*.

Purification of the peptides from frogs of the Cape Peninsula range

The pooled skin secretions from the Cape Peninsula frogs, after partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 preparative reversed-phase HPLC column (Fig. 1). The prominent peaks designated 1–16 were collected and subjected to further purification. Subsequent structural analysis demonstrated that peak 1 contained magainin-G1(1–21),

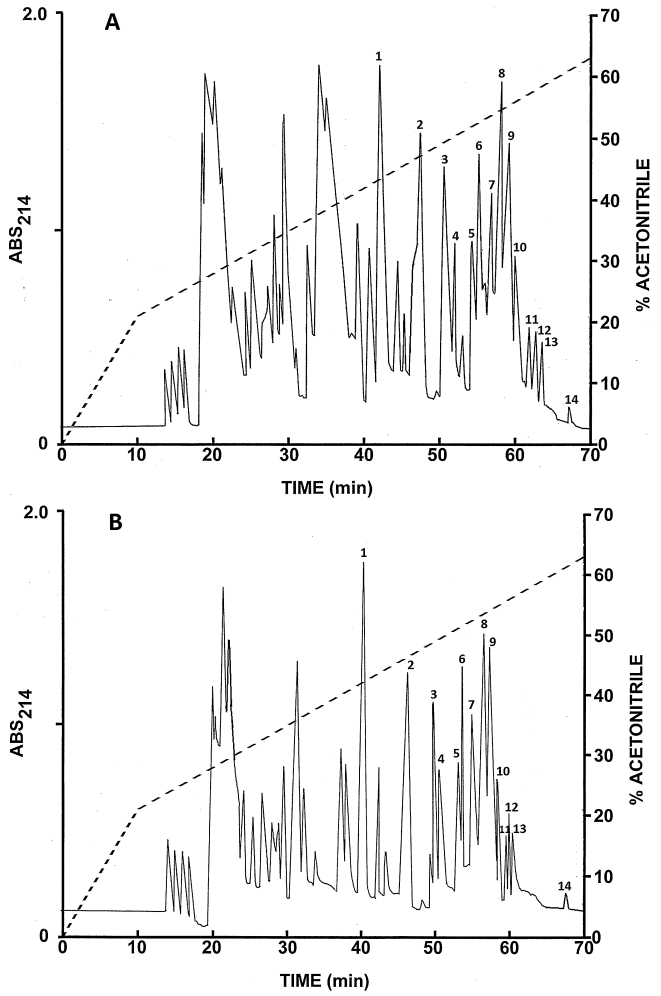

Fig. 2. Reversed-phase HPLC on a preparative Vydac C-18 column of skin secretions from (A) a female and (B) a male *X. gilli* from the Kleinmond range after partial purification on Sep-Pak cartridges. The peaks designated 1–14 contained host-defense peptides and were purified further. The dashed line shows the concentration of acetonitrile in the eluting solvent.

peak 2: magainin-G1, peak 3: magainin-G2, peak 4: PGLa-G1 + PGLa-G2, peak 5: XPF-G1, peak 6: XPF-G2, peak 7: CPF-G1, peak 8: CPF-G2, peak 9: CPF-G3, peak 10: CPF-G4, peak 11: CPF-G5, peak 12: CPF-G6, peak 13: CPF-G7, peak 14: CPF-G8, peak 15: CPF-G9, and peak 16: PGQ-G1. The peptides were purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry, by further chromatography on semipreparative Vydac C-4 and Vydac C-8 columns (chromatograms not shown). The approximate final yields of the more abundant purified peptides (nmol), isolated from 8 frogs, were magainin-G1 1655, PGLa-G2 440, XPF-G1 205, CPF-G2 30, CPF-G3 230, CPF-G4 105, CPF-G5 45, and CPF-G6 490. The yields of all other peptides were <30 nmol.

Purification of the peptides from frogs of the Kleinmond range

The skin secretions from the female Kleinmond frog, after partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 preparative reversed-phase HPLC column using the same experimental conditions as for secretions from the Cape Peninsula frogs (Fig. 2A). The prominent peaks designated 1–14 were collected and subjected to further purification. Subsequent structural analysis demonstrated that peak 1contained magainin-G1, peak 2: PGLa-G1 + PGLa-G2, peak 3: PGLa-G3 + CPF-G5(1–17), peak 4: CPF-G2(1–17), peak 5: XPF-G3, peak 6: CPF-G2, peak 7: CPF-G3, peak 8: CPF-G5, peak 9: CPF-G6, peak 10: CPF-G10, peak 11: CPF-G11, peak 12: CPF-G9, peak 13: CPF-G12, and peak 14: PGQ-G1. The peptides were purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry, by further chromatography on semipreparative Vydac C-4 and Vydac C-8 columns (chromatograms not shown).

The elution profile of skin secretions from the male Kleinmond frog obtained under the same conditions of chromatography used for the female frog is shown in Fig. 2B. A comparison of this chromatogram with that shown in Fig. 2A indicates that, while the two profiles are not completely superimposable, there is no major difference in the distribution of the peaks in the region of the chromatogram in which the host-defense peptides are eluted (between 40 and 70 min). Peptides from the male frog were not analyzed further.

Structural characterization

The primary structures of the antimicrobial peptides isolated from skin secretions were established by automated Edman degradation and their complete primary structures are shown in Fig. 3. MALDI-TOF mass spectrometry was used to confirm the proposed structures and to demonstrate that PGLa-G1, -G2, and -G3 are C-terminally α-amidated. It is probable that the truncated peptides magainin-G1(1–21), CPF-G2(1–17) and CPF-G5(1–17) represent fragments arising from proteolytic cleavage of the corresponding larger peptides rather than the products of separate genes. CPF-G2 differs from CPF-G3, CPF-G7 differs from CPF-G8, and CPF-G11 differs from CPF-G12 by the single substitution Gln → Glu. It is possible that this change in amino acid sequence occurred through artifactual hydrolysis of the glutamine residue during the collection and purification procedures. However, CPF-G3 containing a C-terminal Glu residue was isolated in much higher yield than CPF-G2 containing a C-terminal Gln residue suggesting that the peptides are probably products of separate genes. It should be pointed out that it is not possible to differentiate EQ from QE at C-terminus of a peptide by MALDI-TOF mass spectrometry or amino acid composition analysis and so there is some uncertainty in the assignment of EQ at the C-terminus of CPF-G3, -G8, -G10, and -G11 as the amounts of the amino acid phenylthiohydantoin derivatives detected were very low.

Antimicrobial and hemolytic activities

The abilities of the most abundant peptides in the skin secretions of the Cape Peninsula frogs to inhibit the growth of reference strains of the Gram-positive bacterium *S. aureus* and the Gram-negative bacterium *E. coli* are compared in Table 1. The CPF peptides CPF-G4 and CPF-G6 were the most potent against both microorganisms, particularly *S. aureus* (MIC = 10 μM) but showed moderately high

Table 1
Minimum inhibitory concentrations (μM) against microorganisms and hemolytic activities (LC_{50} (μM) against human erythrocytes of the endogenous peptides isolated from skin secretions of *X. gilli* from the Cape Peninsula range.

	E. coli	*S. aureus*	LC_{50}
Magainin-G1	80	>80	>160
PGLa-G2	40	80	>160
XPF-G1	80	>80	>160
CPF-G2	80	20	ND
CPF-G3	80	20	85
CPF-G4	40	10	35
CPF-G5	80	10	ND
CPF-G6	40	10	60

ND: not determined.

		Cape Peninsula	Kleinmond	
		M_r obs	M_r obs	M_r calc
Magainin-G1(1-21)	GIGKFLHSAGKFGKAFVGEIM	2194.2	ND	2194.2
Magainin-G1	GIGKFLHSAGKFGKAFVGEIMKS	2409.5	2409.3	2409.3
Magainin-G2	GIKEFVHSLGKFGKAFVGGILNQ	2446.5	ND	2446.4
PGLa-G1	GMASKAGAIAGKIAKVALKAL[a]	1968.2	1968.3	1968.2
PGLa-G2	GMASKAGAIAGKIAKVALKAV[a]	1954.6	1954.2	1954.2
PGLa-G3	GMASKAGAIAGKLAKVAIKAAL[a]	ND	2039.4	2039.2
XPF-G1	GWASKIGQTLGKMAKVGLQELIQPK	2681.5	ND	2681.5
XPF-G2	GWASKIGQTLGKMAKVGLEELIQPK	2682.4	ND	2682.5
XPF-G3	GWASKIGETLGKMAKVGLEELIEPK	ND	2684.7	2684.5
CPF-G1	GFGSFLGKALKAGLKIGANLLGGAPQQ	2613.6	ND	2613.5
CPF-G2	GFGSFLGKALKAALKIGANALGGAPQQ	2585.3	2586.5	2585.5
CPF-G2(1-17)	GFGSFLGKALKAALKIG	ND	1678.2	1678.0
CPF-G3	GFGSFLGKALKAALKIGANALGGAPEQ	2586.7	2587.4	2586.5
CPF-G4	GFGSVLGKLFKTAVKIIPSLLPSKQE	2757.5	ND	2757.6
CPF-G5	GLASFLGKALKAGLKIGANLLGGAPQQ	2593.1	2594.3	2593.5
CPF-G5(1-17)	GLASFLGKALKAGLKIG	ND	1643.9	1644.0
CPF-G6	GFGSFLGKALKAALKVGANMLGGAPQQ	2631.6	2631.8	2631.4
CPF-G7	GLASLLGKALKAGLKIGANLLGGAPQQ	2559.5	ND	2559.5
CPF-G8	GLASLLGKALKAGLKIGANLLGGAPEQ	2560.5	ND	2560.5
CPF-G9	GFGSFLGKALKAALKVGADMLGGAPQQ	2632.4	2632.4	2632.4
CPF-G10	GFGSFLGKALKAALKVGADMLGGAPEQ	ND	2633.2	2633.4
CPF-G11	GLASFLGKALKAGLKIGADLLGGAPEQ	ND	2595.5	2595.8
CPF-G12	GLASFLGKALKAGLKIGADLLGGAPEE	ND	2596.8	2596.8
PGQ-G1	GVLSNIVGYLKKLGTGVVSNLLQQ	2500.8	2500.6	2500.4

ND not detected

Fig. 3. Amino acid sequences, observed molecular masses (M_r obs), and calculated molecular masses (M_r calc) of the host-defense peptides isolated from skin secretions of female *X. gilli* from the Cape Peninsula and Kleinmond ranges.

hemolytic activity against human erythrocytes (LC_{50} = 35 μM for CPF-G4 and LC_{50} = 60 μM for CPF-G6).

Discussion

This study has provided support for the claim that populations of *X. gilli* occupying non-contiguous ranges on opposite sides of False Bay have undergone appreciable genetic and phenotypic divergence. Peptidomic analysis has been used to identify and characterize multiple peptides belonging to magainin, PGLa, CPF, and XPF families from skin secretions of frogs inhabiting both ranges. In addition, a peptide with limited amino acid sequence similarity to peptide glycine–glutamine (PGQ), previously identified only in an extract of the stomach of *X. laevis* [32], was isolated from secretions of frogs from both ranges. *X. laevis* PGQ shows broad-spectrum, albeit weak, antimicrobial activity [32]. A comparison of the chromatograms of skin secretions of female frogs from the Cape Peninsula (Fig. 1) and Kleinmond (Fig. 2A) ranges reveals definite differences in the distribution of host-defense peptides. Although the female *X. gilli* from both the Cape Peninsula and Kleinmond ranges are significantly larger than males in the same population, comparison of the chromatograms shown in Fig. 2A (Kleinmond female) and Fig. 2B (Kleinmond male) does not indicate any major sexual dimorphism in the distribution of peptides in skin secretions of this species. Comparing of the primary structures of the host-defense peptides from frogs from both ranges (Fig. 3) confirms that the two populations are associated with an appreciably different spectrum of these peptides. Magainin-G2, XPF-G1, XPF-G2, CPF-G1, CPF-G4, CPF-G7, and CPF-G8 are present in secretions from the Cape Peninsula frogs but were not identified in the skin secretions from Kleinmond frogs. Conversely, PGLa-G3, XPF-G3, CPF-G10, CPF-G11, and CPF-G12 are present in secretions from the Kleinmond frogs but not from the Cape Peninsula animals.

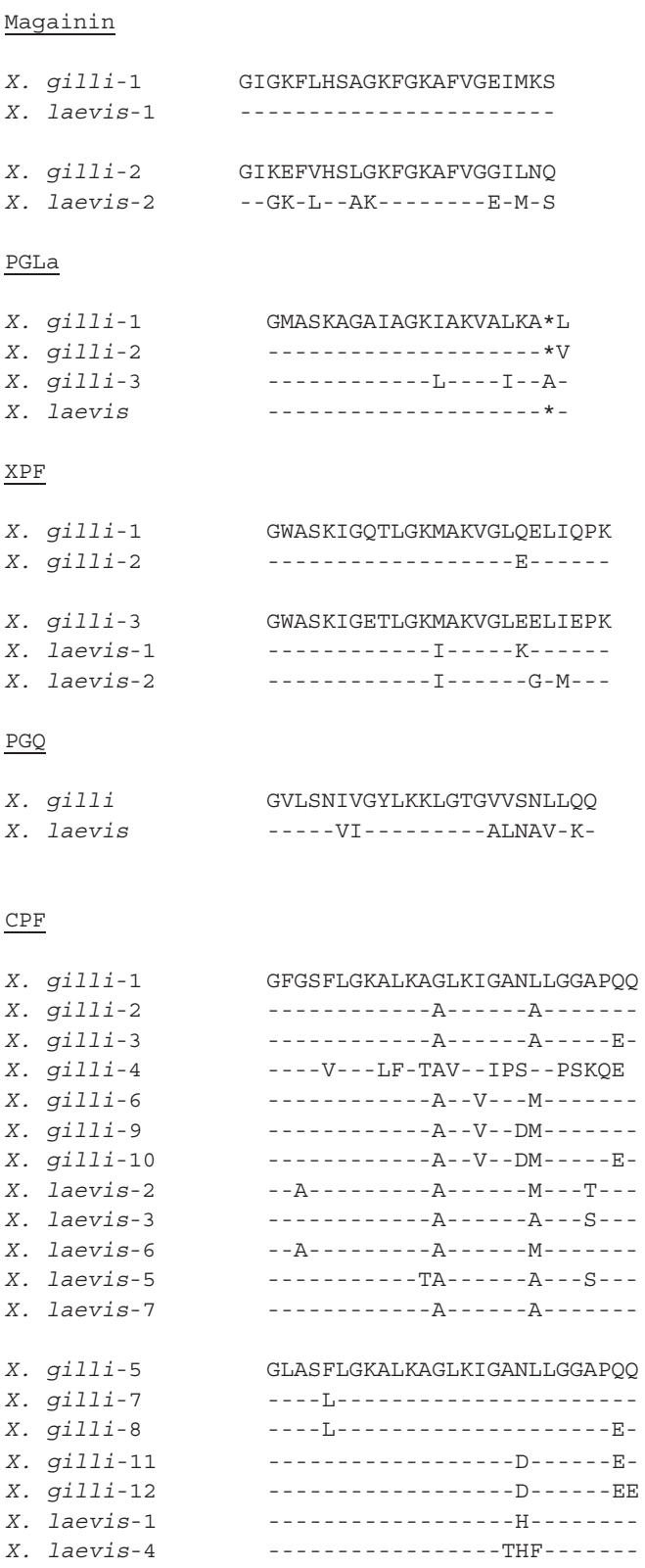

Fig. 4. A comparison of the primary structures of the magainin, PGLa, XPF, PGQ, and CPF peptides from *X. gilli* and *X. laevis*. Residue deletions denoted by * have been introduced into some sequences to maximize sequence similarity.

The study provides further evidence that the amino acid sequences of host-defense peptides in skin secretions can be used, together with morphological analyses and comparisons of nucleotide sequences of mitochondrial and/or nuclear genes, to infer evolutionary relationships among frog species within a particular genus. Largely on the basis of morphology and advertisement calls *X. gilli* has been placed in a species group that also includes the tetraploid frogs *X. laevis*, *Xenopus largeni*, *X. petersii*, and *X. victorianus* [21]. A mitochondrial DNA phylogeny has placed *X. gilli* as sister-group to *X. laevis* [11] although it was

subsequently pointed out that *X. laevis* contains substantially differentiated populations that may warrant separate species status [10]. The primary structures of the host-defense peptides from *X. gilli* identified in this study are compared with those of the corresponding peptides from *X. laevis* [29] in Fig. 4. A close phylogenetic relationship with *X. laevis* is supported by similarities in the distribution and amino acid sequences of orthologous peptides but only three peptides (magainin-G1/magainin-1, PGLa-G1/PGLa, and CPF-G7/CPF-2) are identical in both species.

Peptidomic analysis of skin secretions has also been shown to be of value in differentiating between sub-species and even different population clusters of the same species. The present study complements an earlier investigation in which peptidomic analysis of skin secretions was used to show that *X. muelleri* from an East African range in Malawi is not conspecific with an incompletely characterized species from a non-contiguous west African range in Nigeria that has been referred to as "*Xenopus* new tetraploid 1" and provisionally designated *X. muelleri* West [28]. Similarly, it was shown using the same methodology that frogs referred to as *X. victorianus* from Uganda and *X. laevis sudanensis* from Nigeria are more closely related to *X. petersii* individuals collected in Zambia than to laboratory strains of *X. laevis* that originated in South Africa [19].

The Cape Floral Region is recognized as an area of extreme biodiversity in fauna as well as flora. *X. gilli* is only one of several species with disjunct ranges on either side of False Bay [14]. Among the anurans, the endangered western leopard toad *Amietophrynus pantherinus* shares a similar distribution and restricted dispersal ability as *X. gilli* [26]. Molecular phylogeographic studies using the ND2 marker have shown sequence divergence and different haplotype diversity between the two populations and indicate that gene flow across the distribution gap ceased around 12,000 years ago. *A. pantherinus*, like *X. gilli*, is acidophilic and it has been proposed that fragmentation of a once more widespread distribution into non-contiguous zones is a result of climate change [14,26]. Both species presently occur within Cape Flats Sand Fynbos region, which is acidic, while the geographic gap is located in the Cape Flats Dune Strandveld region which is strongly alkaline. Substantial uplifting on the eastern side of South Africa around 5 Mya resulted in significant reductions in rainfall in the west. During a time of low rainfall, pH of water in potential breeding sites would become more strongly influenced by the substrate so that the pools in Cape Flats intervening region would no longer be able to sustain life of the *X. gilli* larvae [14]. This hypothesis was considered to be more plausible than the alternative proposal that fragmentation of the range began as a result of marine incursions at times of high sea-level and continued through anthropogenically mediated habitat change on the Cape Flats [12].

Habitat invasion by *X. laevis* is a global phenomenon and a cause of concern to conservationists worldwide (reviewed in [25]). There have been several reports dating from 1978 of natural hybridization of *X. gilli* and *X. laevis* with which it is sympatric [20,33,35,36]. The resulting male offspring are infertile and the female hybrids have reduced fecundity. The extent to which this hybridization occurs is uncertain. A study reported in 1997 by Evans et al. [13] found only a single hybrid out of 32 *X gilli* sampled in the eastern region and none were found out of 59 sampled in the Cape Peninsula region. It was concluded the *X. laevis* introgression was not the greatest obstacle to *X. gilli* conservation. A later study by Fogell et al. [14] carried out in 2010–2011 found a much higher incidence of hybridization in animals that were identified morphologically as *X. gilli*, rising to 27% in ponds in the Kleinmond region that had become dominated by *X. gilli*. It was suggested, therefore, that the problem of *X. gilli* introgression has become more serious. Lamloch Pond from which the Kleinmond frogs were collected in the present study had undergone a major invasion by *X. laevis*. Out of a total of 74 animals collected on three successive days, 62 were clearly identified as *X. laevis*. However, the identity of all 10 frogs provisionally described as pure *X. gilli* on morphological grounds was confirmed by subsequent genotyping. Only two hybrids with mixed *X. gilli* × *X. laevis* genotype were found. Further studies are ongoing to assess the importance of protecting *X. gilli* habitats from *X. laevis* introgression in the overall conservation strategy for the species [24].

Consistent with previous studies with skin secretions of *X. borealis* [27], *X. muelleri* [28] and *X. petersii* [18], CPF peptides show the greatest potency against bacteria, particularly *S. aureus* (Table 1) but are appreciably hemolytic against human erythrocytes. Over 27 years have passed since the discovery of the magainins in the skin of *X. laevis* [17,40] and, since that time, several hundred peptides have been isolated from the skin secretions of many other frog species belonging to different families [39]. However, it is fair to claim that the initial promise of these peptides as therapeutically valuable anti-infective agents has not been fulfilled. No antimicrobial compound based upon the structure of a frog skin peptide has yet been adopted in clinical practice so that alternative clinical applications are being sought [6]. The CPF peptides from *X. laevis* and *Silurana epitropicalis* stimulate the release of insulin from BRIN-BD11 clonal β-cells at subnanomolar concentrations and so peptides from the CPF family may serve as templates for the design of long-acting, non-toxic analogs for the treatment of patients with type 2 diabetes [38].

Acknowledgments

This work was supported by the Terry Fox Fund for Cancer Research and incentive funding from the National Research Foundation, South Africa. GJM would like to thank Megan Koordom and the DST-NRF Center of Excellence for Invasion Biology. The authors thank Manju Prajeep, United Arab Emirates University for technical assistance.

References

[1] Clinical Laboratory and Standards Institute Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard M07-A8. Wayne, PA: CLSI; 2008.
[2] Conlon JM. Purification of naturally occurring peptides by reversed-phase HPLC. Nat Protoc 2007;2:191–7.
[3] Conlon JM. Structural diversity and species distribution of host-defense peptides in frog skin secretions. Cell Mol Life Sci 2011;68:2303–15.
[4] Conlon JM, Mechkarska M. Host-defense peptides with therapeutic potential from skin secretions of frogs from the family Pipidae. Pharmaceuticals (Basel) 2014;7:58–77.
[5] Conlon JM, Mechkarska M, Kolodziejek J, Nowotny N, Coquet L, Leprince J, et al. Host-defense peptides from skin secretions of Fraser's clawed frog *Xenopus fraseri* (Pipidae): further insight into the evolutionary history of the Xenopodinae. Comp Biochem Physiol D: Genomics Proteomics 2014;12:45–52.
[6] Conlon JM, Mechkarska M, Lukic ML, Flatt PR. Potential therapeutic applications of multifunctional host-defense peptides from frog skin as anti-cancer, anti-viral, immunomodulatory, and anti-diabetic agents. Peptides 2014;57:67–77.
[7] Conlon JM, Mechkarska M, Prajeep M, Sonnevend A, Coquet L, Leprince J, et al. Host-defense peptides in skin secretions of the tetraploid frog *Silurana epitropicalis* with potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Peptides 2012;37:113–9.
[8] Conlon JM, Prajeep M, Mechkarska M, Coquet L, Leprince J, Jouenne T, et al. Characterization of the host-defense peptides from skin secretions of Merlin's clawed frog *Pseudhymenochirus merlini*: insights into phylogenetic relationships among the Pipidae. Comp Biochem Physiol D: Genomics Proteomics 2013;8:352–7.
[9] Evans BJ, Bliss SM, Mendel SA, Tinsley RC. The Rift Valley is a major barrier to dispersal of African clawed frogs (*Xenopus*) in Ethiopia. Mol Ecol 2011;20:4216–30.
[10] Evans BJ, Greenbaum E, Kusamba C, Carter TF, Tobias ML, Mendel SA, et al. Description of a new octoploid frog species (Anura: Pipidae: *Xenopus*) from the Democratic Republic of the Congo, with a discussion of the biogeography of African clawed frogs in the Albertine Rift. J Zool 2011;283:276–90.
[11] Evans BJ, Kelley DB, Tinsley RC, Melnick DJ, Cannatella DC. A mitochondrial DNA phylogeny of African clawed frogs: phylogeography and implications for polyploid evolution. Mol Phylogenet Evol 2004;33:197–213.
[12] Evans BJ, Morales JC, Picker MD, Kelley DB, Melnick DJ. Comparative molecular phylogeography of two *Xenopus* species, *X. gilli* and *X. laevis*, in the south-western Cape Province, South Africa. Mol Ecol 1997;6:333–43.

[13] Evans BJ, Morales JC, Picker MD, Melnick DJ, Kelley DB. Absence of extensive introgression between *Xenopus gilli* and *Xenopus laevis laevis* (Anura:Pipidae) in southwestern Cape province, South Africa. Copeia 1998:504–9.
[14] Fogell DJ, Tolley KA, Measey GJ. Mind the gaps: investigating the cause of the current range disjunction in the Cape Platanna, *Xenopus gilli* (Anura:Pipidae). Peer J 2013;1:e166.
[15] Frost DR. Amphibian species of the world: an online reference. Version 6.0. New York, USA: American Museum of Natural History; 2011. Electronic database accessible at http://research.amnh.org/herpetology/amphibia/index.php
[16] Gibson BW, Poulter L, Williams DH, Maggio JE. Novel peptide fragments originating from PGL[a] and the caerulein and xenopsin precursors from *Xenopus laevis*. J Biol Chem 1986;261:5341–9.
[17] Giovannini MG, Poulter L, Gibson BW, Williams DH. Biosynthesis and degradation of peptides derived from *Xenopus laevis* prohormones. Biochem J 1987;243:113–20.
[18] King JD, Mechkarska M, Coquet L, Leprince J, Jouenne T, Vaudry H, et al. Host-defense peptides from skin secretions of the tetraploid frogs *Xenopus petersii* and *Xenopus pygmaeus*, and the octoploid frog *Xenopus lenduensis* (Pipidae). Peptides 2012;33:35–43.
[19] King JD, Mechkarska M, Meetani MA, Conlon JM. Peptidomic analysis of skin secretions provides insight into the taxonomic status of the African clawed frogs *Xenopus victorianus* and *Xenopus laevis sudanensis* (Pipidae). Comp Biochem Physiol D: Genomics Proteomics 2013;8:250–4.
[20] Kobel HR, Du Pasquier L, Tinsley RC. Natural hybridization and gene introgression between *Xenopus gilli* and *Xenopus laevis laevis*. J Zool 1981;194:317–22.
[21] Kobel HR, Loumont C, Tinsley RC. The extant species. In: Tinsley RC, Kobel HR, editors. The biology of *Xenopus*. Oxford: Clarendon Press; 1996. p. 9–33.
[22] Mariano DO, Yamaguchi LF, Jared C, Antoniazzi MM, Sciani JM, Kato MJ, et al. *Pipa carvalhoi* skin secretion profiling: absence of peptides and identification of kynurenic acid as the major constitutive component. Comp Biochem Physiol C: Toxicol Pharmacol 2014;167C:1–6.
[23] Matthijs S, Ye L, Stijlemans B, Cornelis P, Bossuyt F, Roelants K. Low structural variation in the host-defense peptide repertoire of the dwarf clawed frog *Hymenochirus boettgeri* (Pipidae). PLoS ONE 2014;9:e86339.
[24] Measey GJ, Davies SJ. Struggling against domestic exotics at the southern end of Africa. Froglog 2011;97:28–30.
[25] Measey GJ, Roedder D, Green SL, Kobayashi R, Lillo F, Lobos G, et al. Ongoing invasions of the African clawed frog *Xenopus laevis*: a gobal review. Biol Invasions 2012;14:2255–70.
[26] Measey GJ, Tolley KA. Investigating the cause of the disjunct distribution of *Amietophrynus patherinus*, the endangered South African western leopard toad. Conserv Genet 2011;12:61–70.
[27] Mechkarska M, Ahmed E, Coquet L, Leprince J, Jouenne T, Vaudry H, et al. Antimicrobial peptides with therapeutic potential from skin secretions of the Marsabit clawed frog *Xenopus borealis* (Pipidae). Comp Biochem Physiol C: Toxicol Pharmacol 2010;152:467–72.
[28] Mechkarska M, Ahmed E, Coquet L, Leprince J, Jouenne T, Vaudry H, et al. Peptidomic analysis of skin secretions demonstrates that the allopatric populations of *Xenopus muelleri* (Pipidae) are not conspecific. Peptides 2011;32:1502–8.
[29] Mechkarska M, Meetani M, Michalak P, Vaksman Z, Takada K, Conlon JM. Hybridization between the African clawed frogs *Xenopus laevis* and *Xenopus muelleri* (Pipidae) increases the multiplicity of antimicrobial peptides in skin secretions of female offspring. Comp Biochem Physiol D: Genomics Proteomics 2012;7:285–91.
[30] Mechkarska M, Prajeep M, Leprince J, Vaudry H, Meetani MA, Evans BJ, et al. A comparison of host-defense peptides in skin secretions of female *Xenopus laevis* × *Xenopus borealis* and *X. borealis* × *X. laevis* F1 hybrids. Peptides 2013;45:1–8.
[31] Mechkarska M, Prajeep M, Coquet L, Leprince J, Jouenne T, Vaudry H, et al. The hymenochirins: a family of host-defense peptides from the Congo dwarf clawed frog *Hymenochirus boettgeri* (Pipidae). Peptides 2012;35:269–75.
[32] Moore KS, Bevins CL, Brasseur MM, Tomassini N, Turner K, Eck H, et al. Antimicrobial peptides in the stomach of *Xenopus laevis*. J Biol Chem 1991;266:19851–7.
[33] Picker MD. Hybridization and habitat selection in *Xenopus gilli* and *Xenopus laevis* in the south-western Cape Province. Copeia 1985:574–80.
[34] Picker MD. Embryonic tolerance of *Xenopus* (Anura) to acidic blackwater. Copeia 1993:1072–81.
[35] Picker MD, de Villiers AL. The distribution and conservation status of *Xenopus gilli* (Anura, Pipidae). Biol Conserv 1989;49:169–83.
[36] Rau RE. The development of *Xenopus gilli* Rose & Hewitt (Anura, Pipidae). Ann S Afr Mus 1978;76:247–63.
[37] South African Frog Re-assessment Group (SA-FRoG), IUCN SSC Amphibian Specialist Group. *Xenopus gilli*. The IUCN Red List of Threatened Species. Version 2014.2; 2010. Electronic database accessible at www.iucnredlist.org
[38] Srinivasan D, Mechkarska M, Abdel-Wahab YH, Flatt PR, Conlon JM. Caerulein precursor fragment (CPF) peptides from the skin secretions of *Xenopus laevis* and *Silurana epitropicalis* are potent insulin-releasing agents. Biochimie 2013;95:429–35.
[39] Wang G, Li X, Wang Z. APD2: the updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res 2009;37:D933–7.
[40] Zasloff M, Magainins. a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms and partial cDNA sequence of a precursor. Proc Natl Acad Sci U S A 1987;84:5449–53.