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# Antimicrobial peptides and alytesin are co-secreted from the venom of the Midwife toad, *Alytes maurus* (Alytidae, Anura): Implications for the evolution of frog skin defensive secretions

Enrico König [a,*], Mei Zhou [b], Lei Wang [b], Tianbao Chen [b], Olaf R.P. Bininda-Emonds [a], Chris Shaw [b]

[a] AG Systematik und Evolutionsbiologie, IBU – Fakultät V, Carl von Ossietzky Universität Oldenburg, Carl von Ossietzky Strasse 9-11, 26129 Oldenburg, Germany
[b] Natural Drug Discovery Group, School of Pharmacy, Medical Biology Center, Queen's University, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland, UK

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ABSTRACT

The skin secretions of frogs and toads (Anura) have long been a known source of a vast abundance of bioactive substances. In the past decade, transcriptome data of the granular glands of anuran skin has given new impetus to investigations of the putative constituent peptides. *Alytes obstetricans* was recently investigated and novel peptides with antimicrobial activity were isolated and functionally characterised. However, genetic data for the evolutionarily ancient lineage to which *Alytes* belongs (midwife toads; Alytidae) remains unavailable.

Here we present the first such genetic data for Alytidae, derived via the granular gland transcriptome of a closely-related species of midwife toad, *Alytes maurus*. First, we present nucleotide sequences of the entire peptide precursors for four novel antimicrobial peptides (AMPs). The two precursors resemble those from Bombinatoridae in both their structural architecture and amino acid sequence. Each precursor comprises two AMPs as tandem repeats, with a member of the alyteserin-1 family (alyteserin-1Ma: GFKEVLKADLG SLVKGIAAHVAN-NH2 or alyteserin-1Mb: GFKEVLKAGLGSLVKGIPAHVAN-NH2) followed by its corresponding member from the alyteserin-2 family (alyteserin-2Ma: FIGKLIS AASGLLSHL-NH2 or alyteserin-2Mb: ILGAIIPLVSGLLSHL-NH2). Synthetic replicates of the four AMPs possessed minimal inhibitory concentrations (MICs) ranging from 9.5 to 300 μM, with the most potent being alyteserin-2Ma. Second, we also cloned the cDNA encoding an alytesin precursor, with the active alytesin exhibiting high sequence identity to bombesin-related peptides from other frogs. All putative mature peptide sequences were confirmed to be present in the skin secretion via LC/MS.

The close structural resemblance of the alyteserin genes that we isolated for *A. maurus* with those of *Bombina* provide independent molecular evidence for a close evolutionary relationship between these genera as well as more support for the convergent evolution of the AMP system within anurans. In contrast to the more evolutionarily conserved nature of neuropeptides (including alytesin, which we also isolated), the more variable nature of the AMP system together with the sporadic distribution of AMPs among anuran amphibians

*Abbreviations:* AMPs, antimicrobial peptides; BLPs, bombesin-like peptides; cDNA, complementary deoxyribonucleic acid; CFU, colony forming units; DMSO, dimethyl sulfoxide; GRP, gastrin-releasing peptide; HPLC, high performance liquid chromatography; LTQ, linear ion trap; MALDI-TOF, matrix-assisted laser desorption/ionisation, time-of-flight; MIC, minimal inhibitory concentration; MS, mass spectrometry; NCBI, National Center for Biotechnology Information; OD, optical density; TFA, trifluoroaceticacid.

* Corresponding author. Tel.: +49 441 798 3373; fax: +49 441 798 193965.
*E-mail address:* en.koenig@uni-oldenburg.de (E. König).

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fuels in part our hypothesis that the latter system was co-opted secondarily to fulfil a function in the innate immune system, having originally evolved for defence against potential macropredators.

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## 1. Introduction

The skin secretion of anuran amphibians (frogs and toads) is known to be an extraordinarily rich source of diverse bioactive substances. More than half a century worth of research on several hundred species of frogs and toads, has unveiled a plethora of natural products including alkaloids, steroids (bufadienolids), amines and peptides (Daly et al., 2005; Erspamer, 1994; Pukala et al., 2006). Pharmaceutical studies undertaken over the past few decades have demonstrated the huge potential for drug discovery and medical therapeutics of frog skin compounds among which the biologically active peptides are by far the most promising candidates (Calderon et al., 2011; Clarke, 1997; Conlon et al., 2004; Lazarus and Attila, 1993). These peptides include neuropeptides that act as both neurotransmitters or neuromodulators (e.g., the gastrin-related bombesin, the cholecystokinin-like caeruleins, or the tachykinins, bradykinins and an array of other vasoactives) as well as opioids/dermorphins and antibiotic peptides (also known as antimicrobial peptides or AMPs) (Bevins and Zasloff, 1990).

In this study, we focus on two of these compound classes, the bombesin-like peptides (BLPs) and AMPs and their expression in the skin secretion of the Midwife toad *Alytes maurus*. Our research follows on from the early work of the group of Vittorio Erspamer, one of the pioneers in the field of frog skin peptides, who isolated alytesin and bombesin from the two closely-related species *Alytes obstetricans* (Alytidae) and *Bombina bombina* (Bombinatoridae), respectively (Anastasi, 1971). Since then, the skin secretions of several *Bombina* species have been investigated intensively and numerous substances have been characterised, including bradykinin-related peptides (Yasuhara et al., 1973), amines (Erspamer, 1994) and numerous AMPs (Simmaco et al., 2009). However, no further research was carried out on midwife toads until the recent identification of alyteserins from *A. obstetricans*, the first peptides with antimicrobial activity known from Alytidae (Conlon et al., 2009a).

Both BLPs and AMPs are widely distributed among anurans. The co-occurrence of AMPs whenever BLPs are present is an intriguing fact, and one that can apparently be generalised to neuroactive peptides, which appear to be always accompanied by AMPs in various frog species (Fig. 1). Otherwise, however, their potential defensive mode of action is quite different. The structural counterparts of the neuroactive BLPs in other amphibian tissues and in other vertebrates – neuromedin B and gastrin-releasing peptides – can stimulate or suppress gastrointestinal secretion, act directly on extravascular smooth muscles, and cause potent antidiuretic effects (Spindel, 2006). In the central nervous system, BLPs can modify thermoregulation, satiety and dipsogenia (Spindel, 2006). Such potent pharmacological properties make BLPs very efficient defensive agents against predator attack, especially given that the major predators of frogs are other vertebrates (e.g., snakes, birds, or mammals). In addition to *Bombina* and *Alytes*, BLPs also occur in skin secretions of other anurans including species of the genera *Litoria* (litorin), *Phyllomedusa* (phyllolitorin) and *Rana* (ranatensin) (Anastasi et al., 1975; Barra et al., 1985; Nakajima et al., 1970; Spindel, 2006; Yasuhara et al., 1983).

Because BLPs, together with other neuroactive peptides and their vertebrate structural counterparts, act on specific receptors in a stereo-specific manner, they tend to be under high selective pressure which is reflected in their more stringently-conserved amino acid sequences [unpublished observations: König, Clark, Shaw]. However, convergent evolution in the form of gene duplication also seems to have played an important role in this class of peptide toxins (Roelants et al., 2010; Wang et al., 2009). As indicated by studies on *Bombina orientalis*, the gastrin-releasing peptides (GRP) from intestine/brain and bombesin from skin secretion, although structurally similar to one another, are products of different genes. Both genes are expressed in the brain and stomach, but only that for bombesin is expressed in the skin (Nagalla et al., 1992; Spindel, 2006), suggesting that the latter tissue is the place of production for specialised neuroactive peptides in anurans that probably derive from gene duplication before the radiation of the group as a whole and were subsequently incorporated as a defensive weapon.

By contrast, despite their similar mode of action to one another, AMPs show a high degree of diversity among anurans. AMPs are united by their amphipathic and cationic nature, which enables them via electrostatic interaction to disrupt the integrity of the negatively charged lipids in the membrane of a target cell with their ability to adopt α-helical structures in membrane environments eventually causing pore formation and lysis (Matsuzaki, 1999). Because this mechanism is very similar to the actions of the complement system of other vertebrates, amphibian AMPs have been considered to be part of the amphibian innate immune system (Nicolas and Mor, 1995; Woodhams et al., 2007). According to this theory, AMPs evolved initially to counteract the permanent threat of potential infections posed by the moist, mucous amphibian skin, which provides an optimal breeding environment for microorganisms. Further support derives from the finding that the physiological active products of the AMP gene undergo positive selection (Tennessen, 2005) as would be expected for immune system components.

However, AMPs present a complex distribution across anurans and apparently do not occur in all frog species (Conlon, 2010; Conlon et al., 2009b) (Fig. 1). Closely related species do tend to share a common set of structurally-related AMPs that can be classified into distinct families (Amiche et al., 2008; Conlon, 2008a,b), leading to the hypothesis

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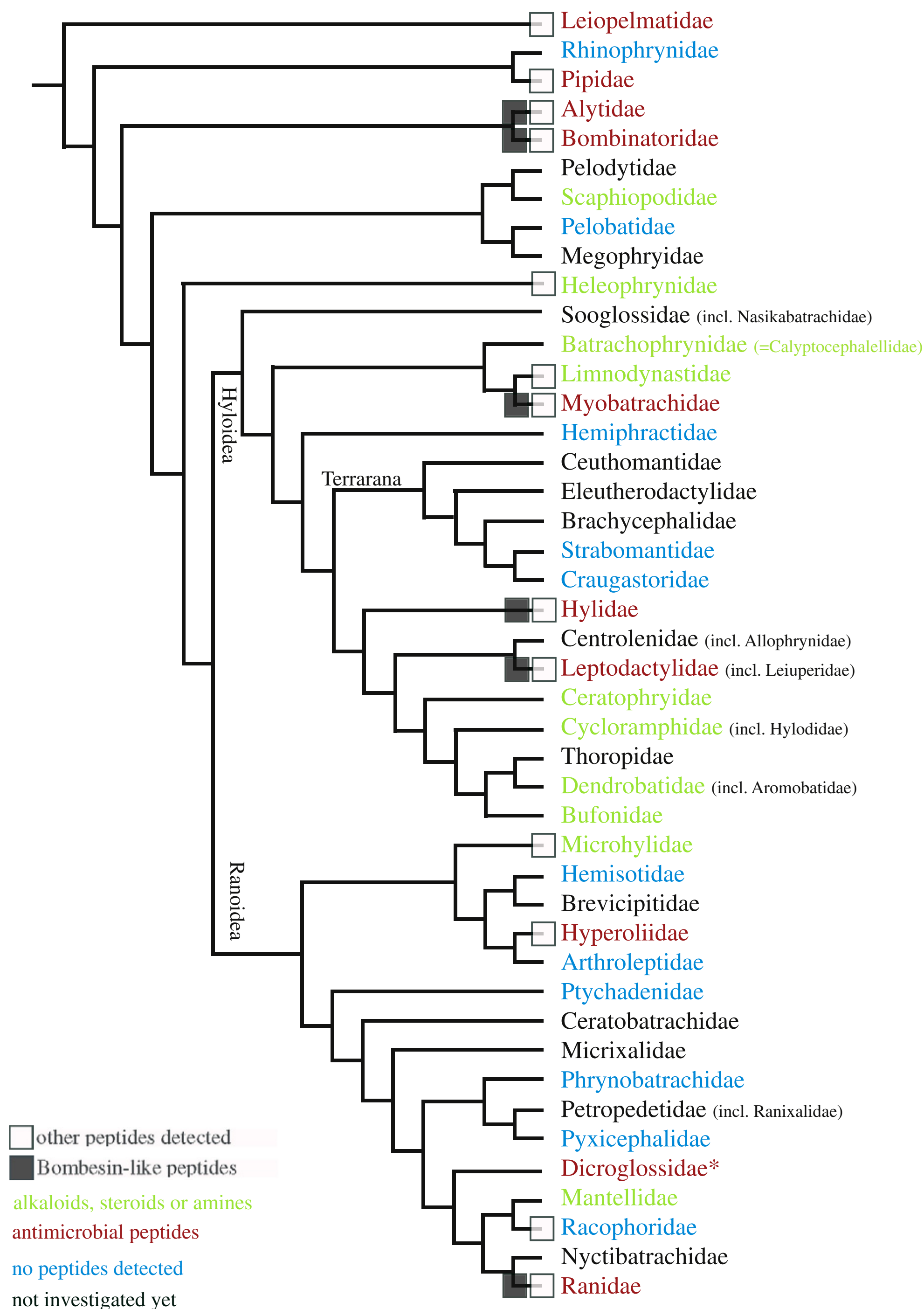


**Fig. 1.** Phylogeny of anuran amphibians (from Frost et al., 2006) and distribution of skin compounds. Note that all taxa from which skin AMPs have been isolated, other peptides and amines have been found (* except for Dicroglossidae).

that very closely related (congeneric) species possess very similar genes for AMP expression. However, this is not absolute and AMPs can be differentially present among close relatives. For example, closely related but geographically isolated genera from the family Pipidae either possess (*Xenopus*, *Silurana*) (Ali et al., 2001; Mechkarska et al., 2011) or lack AMPs (*Pipa pipa*) (Conlon et al., 2009b) in their skin. Another example is Hylidae, where most species of the subfamily Hylinae appear to lack AMPs whereas the South American Phyllomedusinae is the richest sources for amphibian skin AMPs (Calderon et al., 2011).

In addition, expressed AMP genes differ in their architectural structure, which generally comprises a signal sequence followed by an acidic spacer (propiece) and the physiological active peptide (Fig. 2). The signal peptide sequence and the first three amino acids of the propiece are encoded in the first, highly-conserved exon of the antimicrobial peptide gene (Vouille et al., 1997) followed by a variable length intron, and the hypervariable second exon, which encodes the remainder of the propeptide (Duda et al., 2002; Vanhoye et al., 2003). Moreover, the second exon can encode either a single copy (Fleury et al.,

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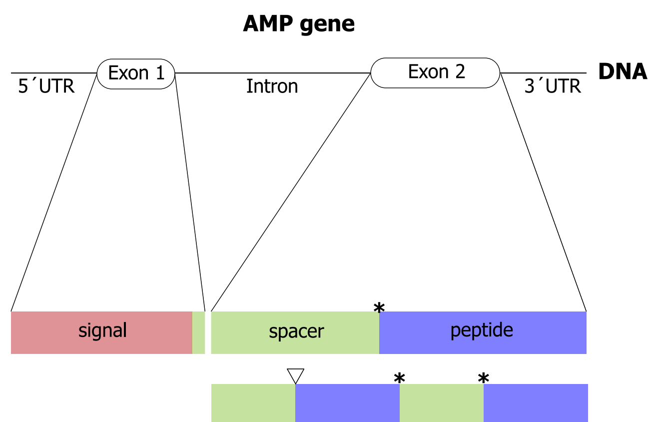


**Fig. 2.** The structural architecture of anuran AMP genes (modified after Miele et al., 1998). In neobatrachian frogs a single copy occurs (upper lane) whereas in Bombinatoridae the second exon contains a tandem repeat of the spacer–peptide part (lower lane). Asterisks designate the -KR- cleavage site and the triangle assigns for the -REIR- cleavage site.

1998; Vanhoye et al., 2003) or a tandem repeat (Miele et al., 1998) of the propeptide. The signal sequences are highly conserved within distantly-related anuran lineages but vary between them providing preliminary evidence that the AMP system might have evolved independently on at least three occasions during the evolution of anuran amphibians (König and Bininda-Emonds, 2011).

In this study, we provide additional information regarding the distribution of BLPs and AMPs in anurans by examining another member of the genus *Alytes*, *A. maurus*, which is the only midwife toad occurring on the African continent with a distribution in the Atlas mountain range of Morocco (Frost, 2011). Here we report four novel peptides with antimicrobial activity isolated from the skin secretion of this species. In addition, using a molecular cloning strategy, we are able to provide the first genetic information for skin peptide-encoding precursors from the family Alytidae, including those for the aforementioned antimicrobial peptides as well as for the neuroactive peptide alytesin. Both sets of data provide additional insight into the evolution and distribution of the respective gene families among anuran amphibians.

## 2. Material and methods

### 2.1. Animals

All experiments with living midwife toads followed both local and national ethical guidelines and were approved by the Veterinary Office of Lower Saxony (permit number 33.9-42502-04-11/0504). Juvenile specimens of *A. maurus* ($n = 5$; 3 male, 2 female) were obtained from a private handler in 2010 and maintained in an appropriate terrarium (60 × 30 × 30 cm) with a water pond and stones that acted as shelter for the animals. A substrate composed of a sand-coconut fibre mix was used to maintain humidity by frequent watering and to facilitate the natural burrowing behaviour for the animals. All individuals were fed three times per week with crickets (*Acheta domesticus*), which were bio-loaded with a vitamin–mineral powder once a week. All animals were maintained at room temperature under a 12/12 h light/dark cycle (10/14 h in winter).

### 2.2. Acquisition of skin secretions

Secretions were obtained from the dorsal skin of the five adult individuals via either a thorough manual massage for several minutes or via mild transdermal electrical stimulation (5 V, 50 Hz, 4 ms pulse width) (Tyler et al., 1992) if the massage did not produce sufficient material. The secretion was then rinsed from the skin surface into a sterile glass beaker with a stream of distilled deionised Milli-Q water. Samples were subsequently snap-frozen using liquid nitrogen before being lyophilised and stored at −20 °C until analysis.

### 2.3. "Shotgun" cloning of *A. maurus* skin secretion-derived cDNA

Five milligrams of lyophilised skin secretion of a single male toad were placed into 1 mL of cell lysis/mRNA stabilisation buffer for 20 min at room temperature. Polyadenylated mRNA was isolated from the buffer using magnetic oligo-dT beads as described by the manufacturer (Dynal Biotech, UK) and subsequently reverse-transcribed using an SMART-RACE kit (Clonetech, UK) to obtain a cDNA library in a two-step process.

In the first step, a sample of the cDNA library was subjected to 3′-RACE PCR using the supplied nested universal primer (NUP) and a degenerate sense primer (S1: 5′-ATHGCNGCNCAYGTNGC-3′) that was designed to previously-reported skin peptides from *A. obstetricans* (Conlon et al., 2009a). The PCR was performed as follows: initial denaturation step: 60 s at 94 °C followed by 35 cycles of denaturation 30 s at 94 °C, primer annealing for 30 s at 54 °C, and extension for 180 s at 72 °C. The resulting PCR fragments of approximately 500 bp were purified (PCR purification Kit, Jena Bioscience, Germany), cloned using a pGEM-T vector system (Promega Corporation) and sequenced by Macrogen (Amsterdam, Netherlands).

In the second step, a specific antisense primer (AS1: 5′-GCARAGCTACCTACTCTGT-3′) was designed according to an apparently conserved site within the 3′-untranslated region of the initial transcript obtained in the previous step. Thereafter, 5′-RACE reactions were performed using the NUP and AS1 primers, with subsequent purification, cloning and sequencing as described above.

The same cDNA library was also employed for molecular cloning of the alytesin precursor using the same two-step procedure as described with a degenerate sense primer (S2: 5′-CARTGGGCNGTNGGNCA-3′) and a specific antisense primer (AS2: 5′-GGTGCTCTGCGCGGCTCCT-3′).

### 2.4. Peptide purification

The lyophilised skin secretions of a male and a female toad (6.6 mg) were pooled and dissolved in 0.05/99.5 (v/v) trifluoroaceticacid (TFA)/water (1 mL) before being cleared of microparticles and other debris by centrifugation. The

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supernatant was then injected onto a reverse phased HPLC system fitted with an analytic column (Luna C18; 250 × 4.6 mm, Phenomenex, UK) using a gradient formed from 0.05/99.95 (v/v) TFA/water to 0.05/29.95/70 (v/v/v) TFA/water/acetonitrile over 240 min at a flow rate of 1 mL/min. Absorbance was constantly monitored at λ214 nm and all fractions (1 mL) were collected. Aliquots of each fraction (300 μl) were lyophilised and redissolved in phosphate saline buffer (PBS) to determine antimicrobial activity (see below).

### 2.5. Identification and structural characterisation of mature peptides in the skin secretions

All fractions were analysed further using MALDI-TOF on a Voyager DE mass spectrometer (PerSeptive Biosystems, MA, USA) in positive detection mode and using alpha-cyano-4-hydroxycinnamic acid as the matrix. Both the primary structures and amidation status of those fractions containing peptides with observed masses matching those for alytesin and alyteserins as predicted from the cloned prepropeptide sequences were determined using an LTQ Orbitrap XL ETD mass spectrometer.

After establishment of the unequivocal primary structures of the peptides, synthetic replicates were obtained from Coring System Diagnostix GmbH (Gernsheim, Germany) where they were purified to at least 95% purity with molecular masses verified by MALDI-TOF and used to facilitate accurate antimicrobial assays (see below).

### 2.6. Antimicrobial assay

We determined those fractions with antimicrobial activity using an agar diffusion test. MICs of synthetic replicates of the identified AMPs (alyteserin-1Ma, -1Mb, -2Ma and -2Mb) were subsequently assessed by liquid microtiter dilution assay using a standard Gram-positive (*Staphylococcus aureus*; NCTC 10788) and Gram-negative bacterium (*Escherichia coli*; NCTC 10418), and a standard pathogenic yeast (*Candida albicans*; NCTC 1467). The synthetic peptides were initially dissolved as a stock solution of 10 mg/mL (4.3–6.2 mM) in dimethyl sulfoxide (DMSO) and subsequently diluted in Mueller–Hinton broth (100/150 v/v). Microorganism cultures ($10^5$ colony forming units (cfu)/mL) were inoculated with peptide concentrations ranging from 1000 to 0.5 μg/mL and placed into 96-well microtiter cell culture plates for incubation at 37 °C (*E. coli*, *S. aureus*) or 30 °C (*C. albicans*) for 20 h in a humidified atmosphere. Following the incubation period, the growth of the respective microorganisms was quantified by measuring their optical density (OD) at 570 nm using an ELISA plate reader (Biolise BioTek EL808). MICs were defined as the lowest concentration of peptide at which no growth was detectable.

### 2.7. Alignment of skin secretion-derived cDNAs and peptide sequences

All full-length cDNA sequences obtained were translated using Expasy (Swiss Institute of Bioinformatics) and identified open-reading frames were then subjected to online BLAST analysis (blastp) at the National Center for Biotechnology Information (NCBI) for preliminary identification and to check for possible contamination. Realignment of the query sequence and resultant hits was performed at the amino-acid level using Clustal W v2.0.10 (Thompson et al., 1994) before manual adjustment.

## 3. Results

### 3.1. "Shotgun" cloning of skin peptide precursor-encoding cDNAs from a skin secretion-derived cDNA library of *A. maurus*

Two different cDNAs encoding the biosynthetic precursors of putative antimicrobial peptides were consistently cloned from the *A. maurus* skin secretion-derived cDNA library. Each open-reading frame consisted of 133 amino acid residues, comprising an N-terminal putative signal peptide followed by a tandem repeat of an acidic propiece peptide in combination with the active AMP domain (Fig. 3A and B). The second propiece is interrupted by two small spacer peptides (of 8 and 10 amino acids in length, respectively), each of which is flanked by four canonical -Lys-Arg- (-KR-) propeptide convertase processing cleavage sites, with the last site being followed immediately by the second active AMP at the C-terminal end of the precursor (Fig. 4A and B).

The open-reading frames of the two AMP domains are 24 and 17 amino acids long, respectively, and show virtually no sequence identity to one another. However, each does possess a C-terminal glycyl residue that acts as the amide donor for the asparaginylamides and leucinamides, respectively, present in the mature peptides (Fig. 4). Each AMP domain also shows high identity to those recently described from the skin secretion of *A. obstetricans* (Conlon et al., 2009a). In accordance with the nomenclature we named the identified AMP homologues, either as alyteserin-1Ma or -1Mb (74%–83% sequence identity to alyteserin-1, respectively) or alyteserin-2Ma and -2Mb (62%–82% to alyteserin-2, respectively) (Fig. 4C). Within the cDNAs, either alyteserin-1Ma and -2Ma or alyteserin-1Mb and -2Mb always occurred as tandem pairs.

The structural architecture of these precursors, where a single signal peptide is followed by tandem duplicates of the remaining components, therefore resembles that of the AMP encoding prepropeptides cloned from the skins of toads from Bombinatoridae (Fig. 4A and B). This includes the first 18N-terminal amino acid residues, which constitute the putative signal sequence (König and Bininda-Emonds, 2011). Comparison of the putative signal sequences from *Bombina* and *Alytes* shows relatively high similarity: 66% identity at the level of amino acid residues and 89% when chemically neutral substitutions are considered (Fig. 4A and B). A notable difference occurs at the N-terminal positions 3 and 4 where *A. maurus* possesses a cysteine and a glutamine residue instead of the respective phenylalanine and lysine residues known for almost all AMP-encoding precursors from *Bombina*, the exception being maximin-S (Wang et al., 2005) with its phenylalanine and asparagine residues. The regions directly flanking the first (internal) peptide domain (i.e.,

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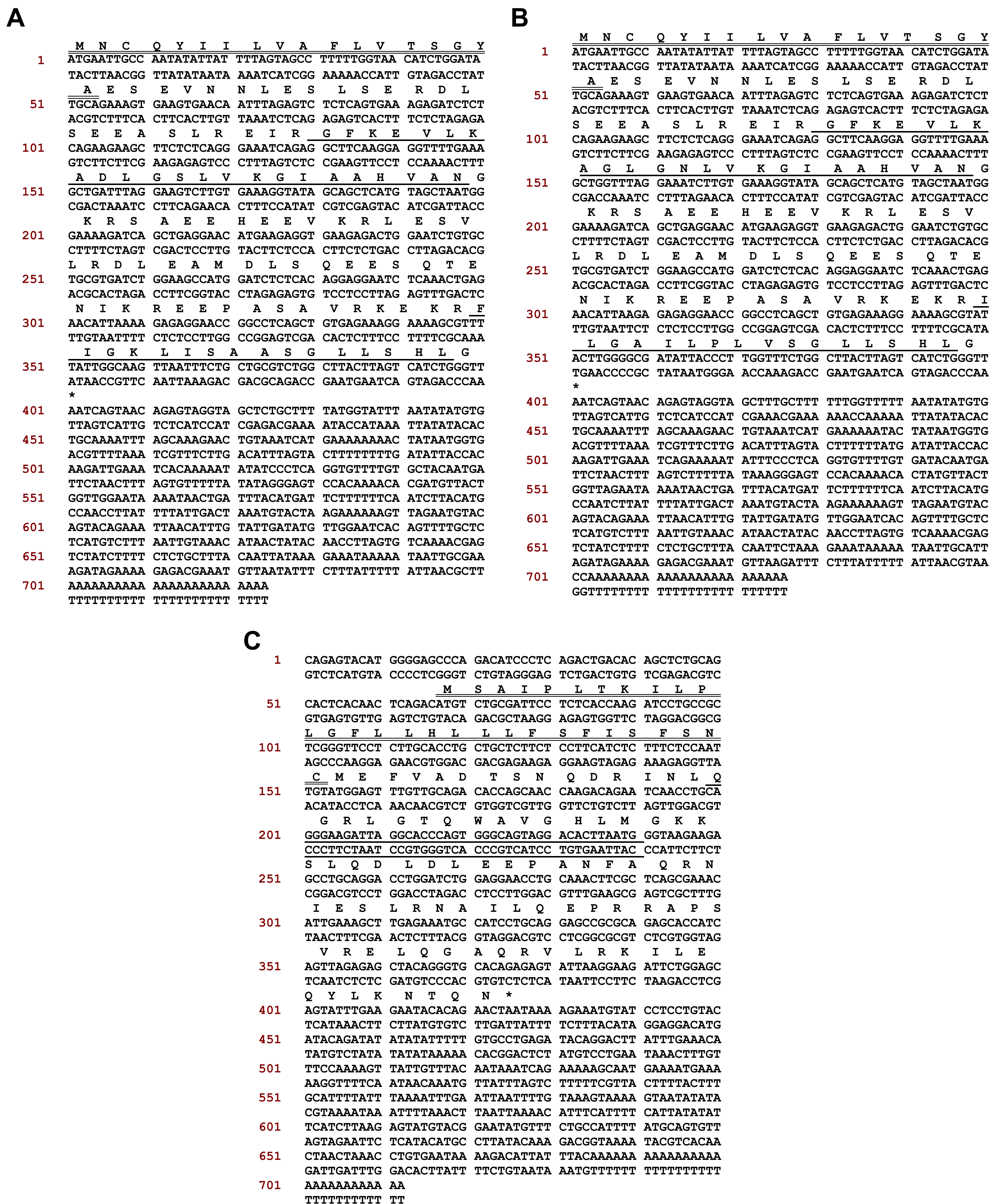


**Fig. 3.** Nucleotide and translated open-reading amino acid sequences of cloned cDNAs encoding the biosynthetic precursors of alyteserin-1Ma/2Ma (A), alyteserin-1Mb/2Mb (B), and alytesin (C). The mature peptides are single-underlined, putative signal sequences are double-underlined, and asterisks designate stop codons.

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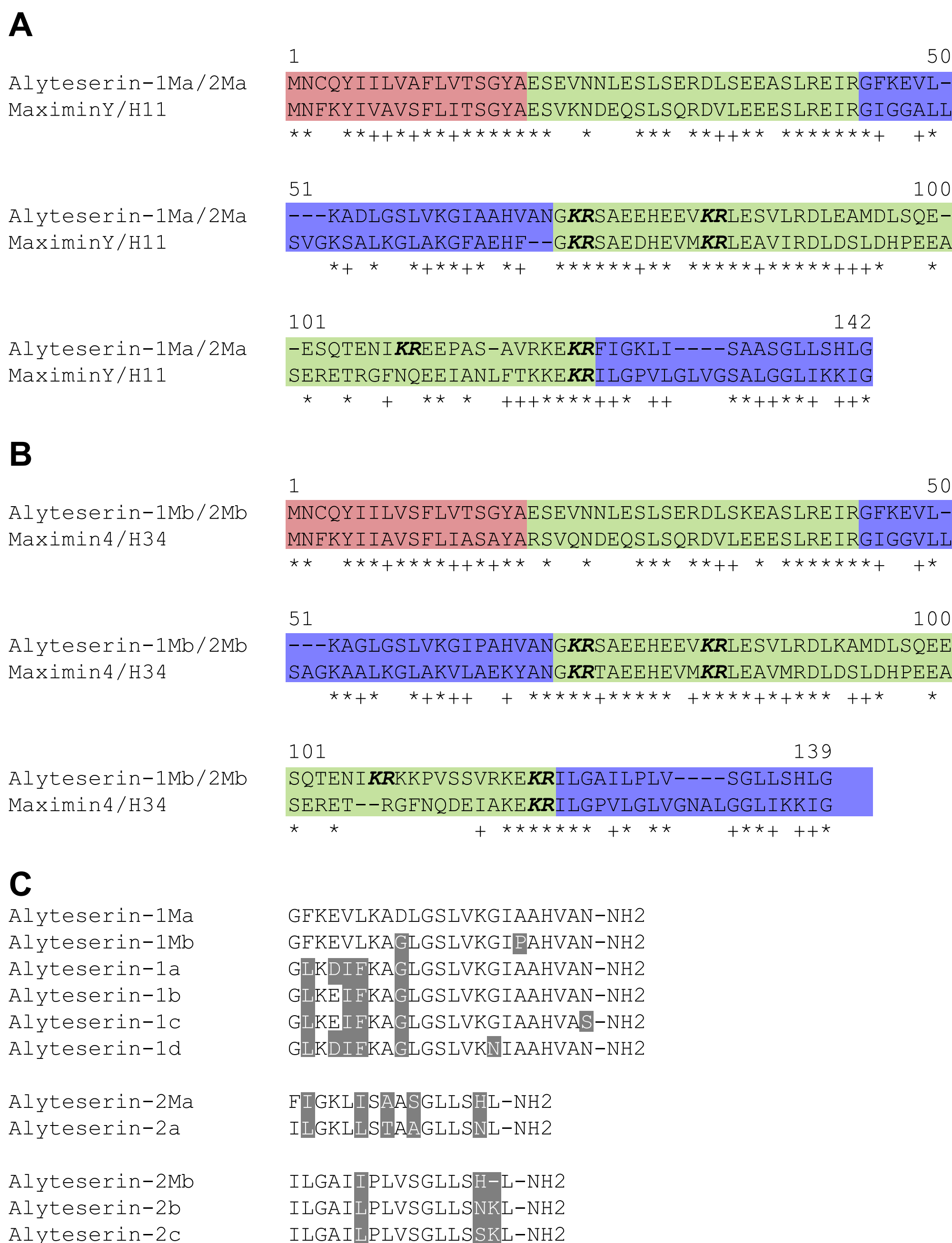


**Fig. 4.** Alignments of prepro-alyteserins, prepro-maximins (A and B), and mature alyteserins (C) isolated from *Alytes maurus*, *A. obstetricans* and *Bombina maxima*, respectively. Colouration in A and B follows that of Fig. 2, substitutions in C are grey boxed. The convertase processing cleavage sites (KR) are in bold italics. Asterisks designate identical amino acid residues and + shows chemically similar substitutions. Accession numbers from NCBI are stated in the text.

alyteserin-1Ma or -1Mb) are also highly conserved when prepro-alyteserin and prepro-bombinins are compared.

BLASTp analyses of the entire precursor open-reading frames against GenBank resulted in several hits with prepro-maximins from *Bombina maxima* (note that according to AMP nomenclature (Amiche et al., 2008; Conlon, 2008a,b) the maximins should be named bombinins). No corresponding molecular data for alyteserin precursor structures from *A. obstetricans* is available in GenBank. Maximum degree of identity at the amino acid level was 49% for alyteserin-1Ma/2Ma (with maximin Y/H11; accession number Q58T95) and 51% for alyteserin-1Mb/2Mb (with maximin 4/H34; Q58T42) (Fig. 4A and B).

From the same cDNA library, we also successfully cloned a cDNA encoding the biosynthetic precursor of the BLP alytesin (Fig. 3C). A comparison of its open-reading frame with those encoding BLPs from other anuran species revealed 71%, 64% and 53% sequence identity at the amino acid level with prepro-bombesin from *B. orientalis*, *Bombina variegata*, and *Lithobates catesbeianus*, respectively (Fig. 5). In addition, precursors encoding neuromedin-B (from *Silurana tropicalis* and *Xenopus laevis*) and ranatensin (from *Lithobates pipiens*) exhibited 49–51% identity to prepro-alytesin from *A. maurus* (Fig. 5).

All sequences from this study have been submitted to GenBank with the following accession numbers: JQ728672

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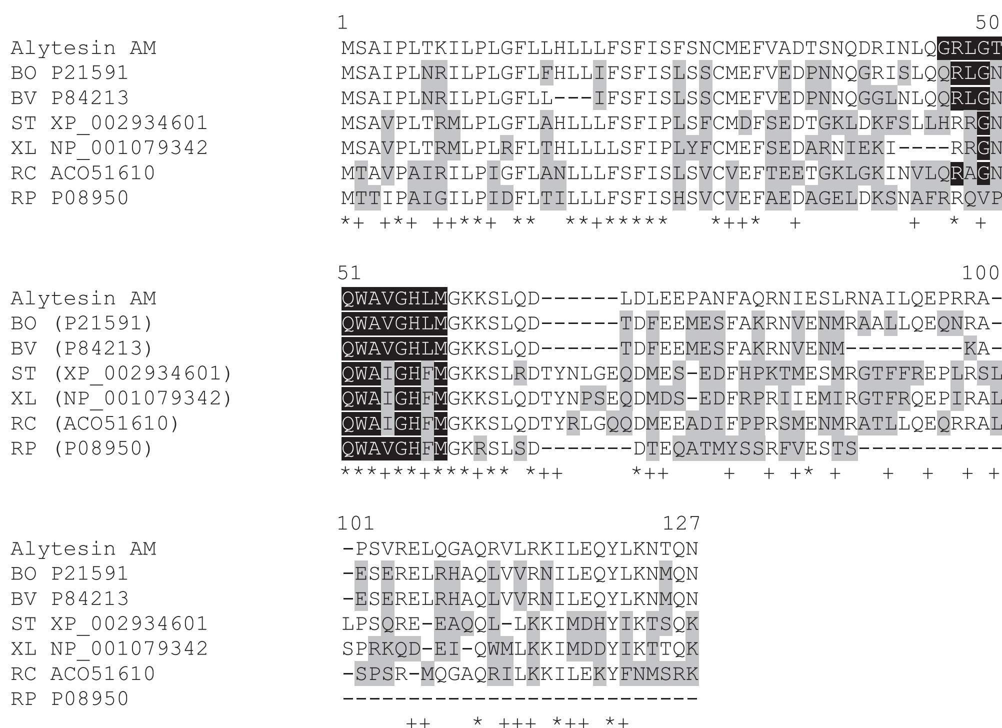


**Fig. 5.** Alignment of cDNA-deduced alytesin precursor sequence with those of other BLPs from different frog species. The active peptide is boxed in black. Substitutions are highlighted in grey. Asterisks designate identical amino acid residues and + shows chemically-similar substitutions. Accession numbers from NCBI are in parentheses. AM: alytesin from *Alytes maurus*, BO: bombesin from *Bombina orientalis*, BV: bombesin from *Bombina variegata*, RC: bombesin from *Rana catesbeiana*, RP: ranatensin from *Rana pipiens*, ST: neuromedin-B from *Silurana tropicalis*, XL: neuromedin-B from *Xenopus laevis*.

for alyteserin-1Ma/2Ma, JQ728673 for alyteserin-1Mb/2Mb, and JQ728674 for prepro-alytesin.

### 3.2. Identification and structural characterisation of mature peptides isolated from the skin secretion of A. maurus

Following reverse phase HPLC fractionation of the skin secretions, a sample of each chromatographic fraction was screened for antimicrobial activity as well as analysed by matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry (MALDI-TOF). Fractions that inhibited the growth of any of *E. coli*, *S. aureus* or *C. albicans* contained peptides with observed molecular masses that matched those predicted from translated cloned cDNAs that encoded the biosynthetic precursors of alyteserins (Table 1). Subsequent MALDI-TOF analyses demonstrated that these fractions contained the peptides alyteserin-1Ma, alyteserin-1Mb, alyteserin-2Ma, and alyteserin-2Mb (peaks #3–6 in Fig. 6). The respective primary structures and amidation status of each peptide were confirmed by MS/MS fragmentation using an LTQ Orbitrap electrospray mass spectrometer (Fig. 7A–D). The net charges of the mature alyteserins from *A. maurus* vary between +1 and +3. The relatively low net positive charge measured for alyteserin-2Mb (+1) therefore explains why the spacer peptide preceding it contains an atypically high number of positively-charged amino acid residues for an AMP precursor (Fig. 4B).

Our analyses also confirm the presence of the BLP alytesin (peak #1 in Fig. 6). An observed $m/z$ ratio of 1533 (MALDI-TOF MS) match the calculated mass of the peptide and its identity as alytesin (pQGRLGTQWAVGHLM-$NH_2$) was absolutely confirmed by MS/MS fragmentation (Fig. 7E and F) and molecular cloning (see above).

In addition, we detected the presence of some lower intensity peptide ions with $m/z$ ratios around 1000 in earlier fractions. These could represent bradykinin-related peptides in the skin secretion of *A. maurus* (data not shown).

### 3.3. Antimicrobial activity of alyteserins

Using a simple agar diffusion test, we first identified five fractions exhibiting significant antimicrobial activity; four of these fractions each contained one of the novel peptides alyteserin-1Ma, -1Mb, -2Ma and -2Mb. The MICs of the synthetic replicates of these novel alyteserins against our model micro-organisms were then subsequently assayed and the results are summarised in Table 2. The inhibitory activity of all alyteserins was of relatively low potency against the eukaryotic yeast *C. albicans*. By contrast, strong activity (MIC 10–20 μM) was observed for the alyteserin-2 family peptides against the Gram-positive *S. aureus* and for alyteserin-2Mb in particular. Alyteserin-2Ma was effective to some degree against all the microorganisms tested, but with a stronger inhibition of both Gram-negative and Gram-positive bacteria. Peptides from the alyteserin-1 family were generally of considerably lower antimicrobial potencies (MIC > 200 μM).

The peptides contained in the fifth fraction (indicated in Fig. 6 as peak #2) were not clearly resolved and were not unequivocally identified (data not shown). However, antimicrobial activity was also observed in this fraction and the molecular masses observed for peptides in this fraction would suggest that they could be additional members of

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**Table 1**
Alyteserins-1 and -2 isolated from skin secretions of *A. maurus*. This table shows the amino acid sequences (one letter code), observed molecular masses (Mr obs), and calculated molecular masses (Mr cal) of the respective peptides.

| Peptide | Fraction | Primary structure | Mr obs (Da) | Mr cal (Da) |
|---|---|---|---|---|
| Alyteserin-1Ma | 129 | GFKEVLKADLGSLVKGIAAHVAN-$NH_2$ | 2337.6 | 2336.8 |
| Alyteserin-1Mb | 138 | GFKEVLKAGLGSLVKGIPAHVAN-$NH_2$ | 2303.8 | 2304.8 |
| Alyteserin-2Ma | 132 | FIGKLISAASGLLSHL-$NH_2$ | 1626.2 | 1626.0 |
| Alyteserin-2Mb | 165 | ILGAIIPLVSGLLSHL-$NH_2$ | 1615.2 | 1615.0 |

the alyteserin-1 family or fragments of a protein with higher molecular weight (*m*/*z* values 2280, 2264, 2305). Noteworthy here is that four different members of the alyteserin-1 family (1a, 1b, 1c, and 1d) and only three different ones from the alyteserin-2 family (2a, 2b, and 2c) were isolated from *A. obstetricans* (Conlon et al., 2009a).

## 4. Discussion

### 4.1. Evolutionary considerations for the anuran AMP system

Our study presents the first full-length cDNA-derived sequences from the skin secretion transcriptome of any member of the family Alytidae. At the amino acid level, both the alyteserins and alytesin sequences we isolated from *A. maurus* show clear homology with their respective counterparts from *A. obstetricans*, highlighting the relatively recent radiation among extant members of the genus (no more than 15-18 MYA (Martínez-Solano et al., 2004)). At the level of prepro-alyteserins our results also indicate a remarkable resemblance with the prepro-bombinins from *B. maxima* in terms of both the general architectural pattern of the transcribed precursors and similarity between the respective signal sequences. For instance, the respective precursors from both species comprise a doubly repeated set of an acidic propiece, a short spacer and a mature peptide following the single putative N-terminal signal sequence. In addition, the internal active peptides from both Alytidae and Bombinatoridae possess an unusual Arg-Glu-Ile-Arg (-REIR-) cleavage site at the N-terminus. Altogether, these similarities support the homology of the precursors and suggest the presence of a common ancestral AMP gene for Alytidae and Bombinatoridae. This, in turn, reflects current phylogenetic opinion placing *Alytes* and *Bombina* in the monophyletic clade Costata on the basis of both larval characters (Haas, 2003) and molecular data (Frost et al., 2006) (Fig. 1). Nevertheless, the long divergence time between these two genera (190 MYA (Roelants et al., 2007)) is reflected by a sequence similarity of only 50% between prepro-alyteserin and prepro-bombinins. Other important differences are also present, including four -KR- cleavage sites in prepro-alyteserin, resulting in two small acidic spacer peptides, versus three in all *Bombina* precursors that consequently exhibit only a single but larger pro-piece following one small acidic spacer peptide. The latter, however, still remarkably resembles that of the alyteserin precursor.

As such, the Costata AMP system is analogous to that documented for Neobatrachia, where the homology of the AMP gene across the group has been postulated based on the common architecture of the transcribed gene (tripartite signal–spacer–peptide structure) and a conserved N-terminal putative signal sequence (König and Bininda-

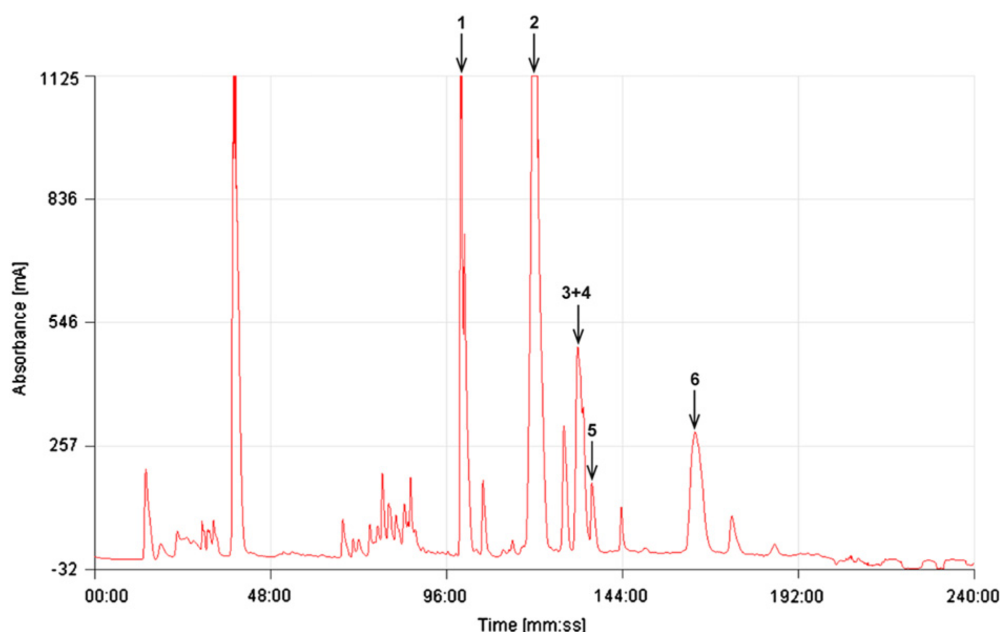


**Fig. 6.** Reverse phase HPLC chromatogram of *Alytes maurus* skin secretion. Numbered arrows indicate fractions with active peptides: 1) alytesin, 3) alyteserin-1Ma, 4) alyteserin-2Ma, 5) alyteserin-1Mb, 6) alyteserin-2Mb. Fractions from peak 2 showed antimicrobial activity, but no peptides have been isolated.

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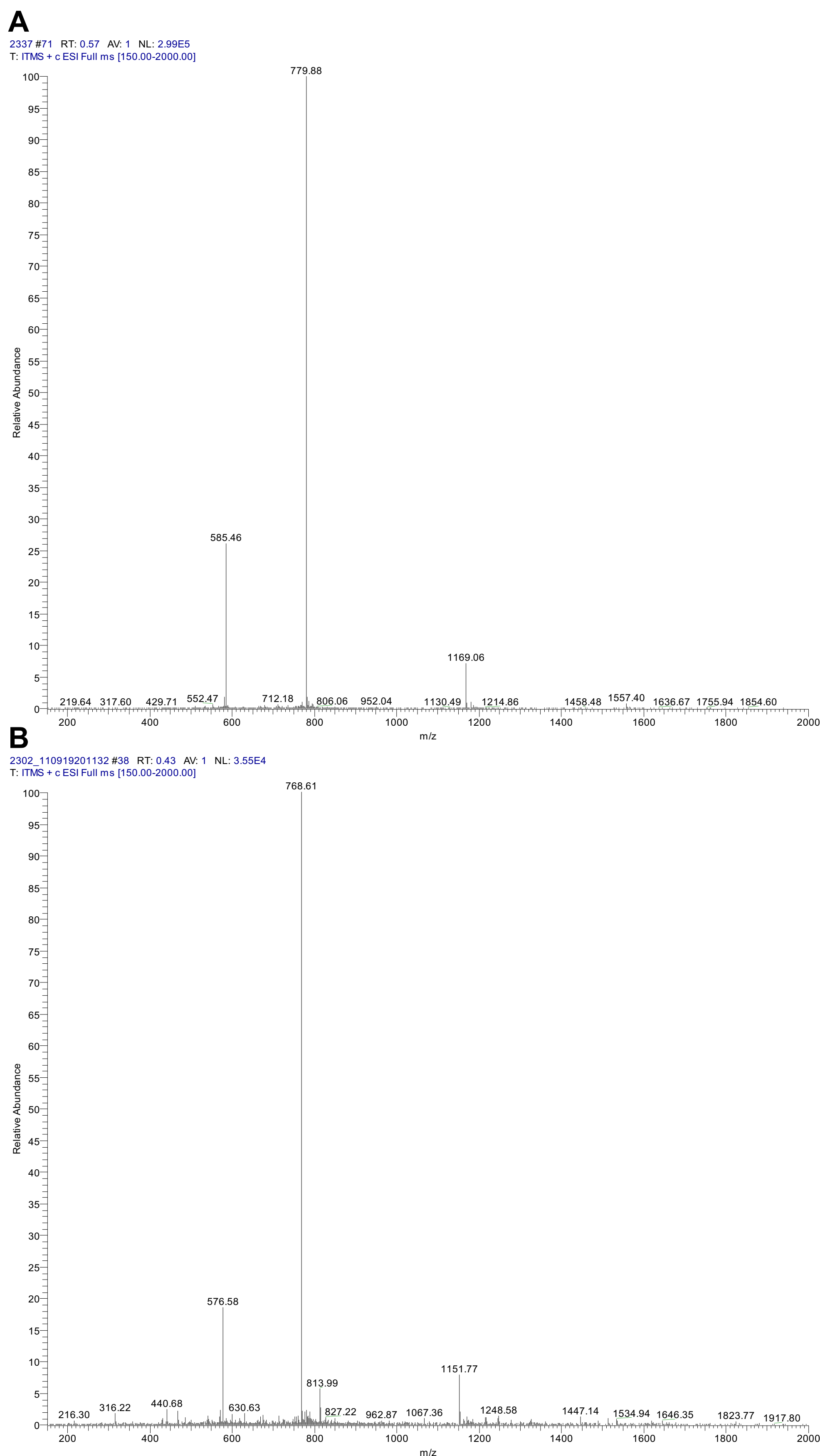


**Fig. 7.** LTQ-Orbitrap mass spectra acquired with the LTQ Orbitrap XL ETD for *Alytes maurus* HPLC fractions. Mass spectra showing the presence of the singly (*$(M + H)^{+}$), doubly (**$(M + 2H)^{2+}$) or triply (***$(M + 3H)^{3+}$) charged ions of (A) alyteserin-1Ma (*m/z* 2337.64), (B) alyteserin-1Mb (*m/z* 2303.83), (C) alyteserin-2Ma (*m/z* 1626.2), and (D) alyteserin-2Mb (*m/z* 1615.22), respectively, are correlated with HPLC fractions displaying antimicrobial activity. The fraction containing alytesin was identified in MALDI-TOF (E) and shows the presence of a singly charged ion (*m/z* 769.1) (F).

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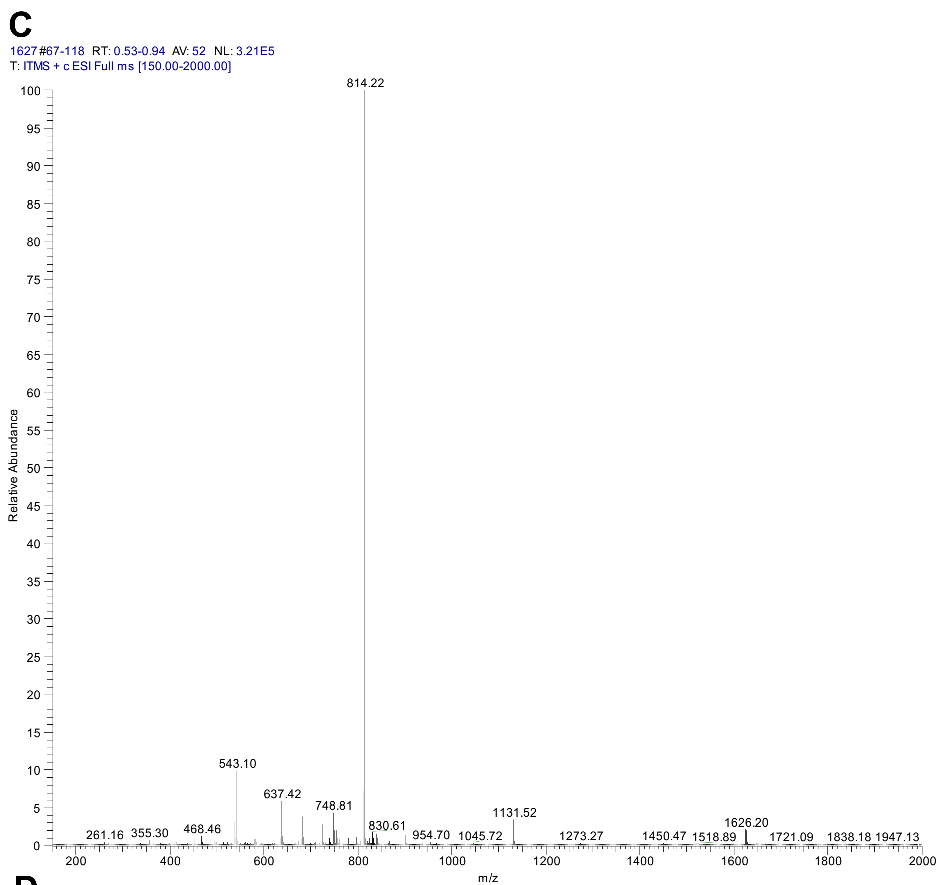


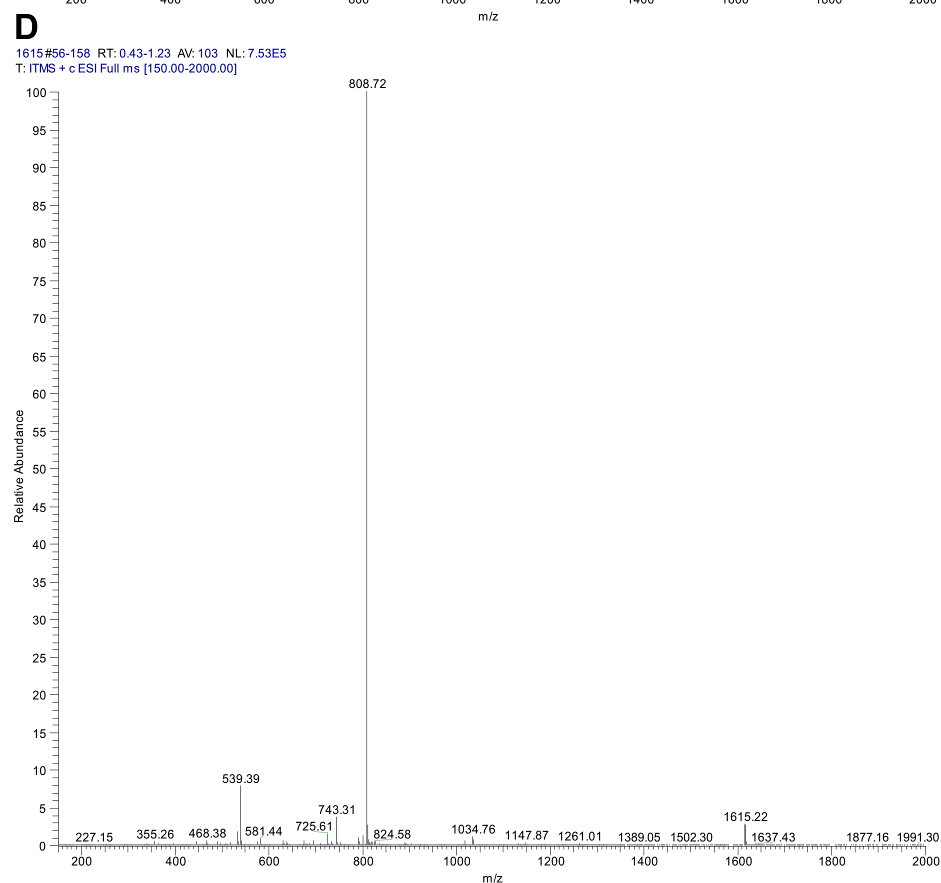


**Fig. 7.** (*continued*).

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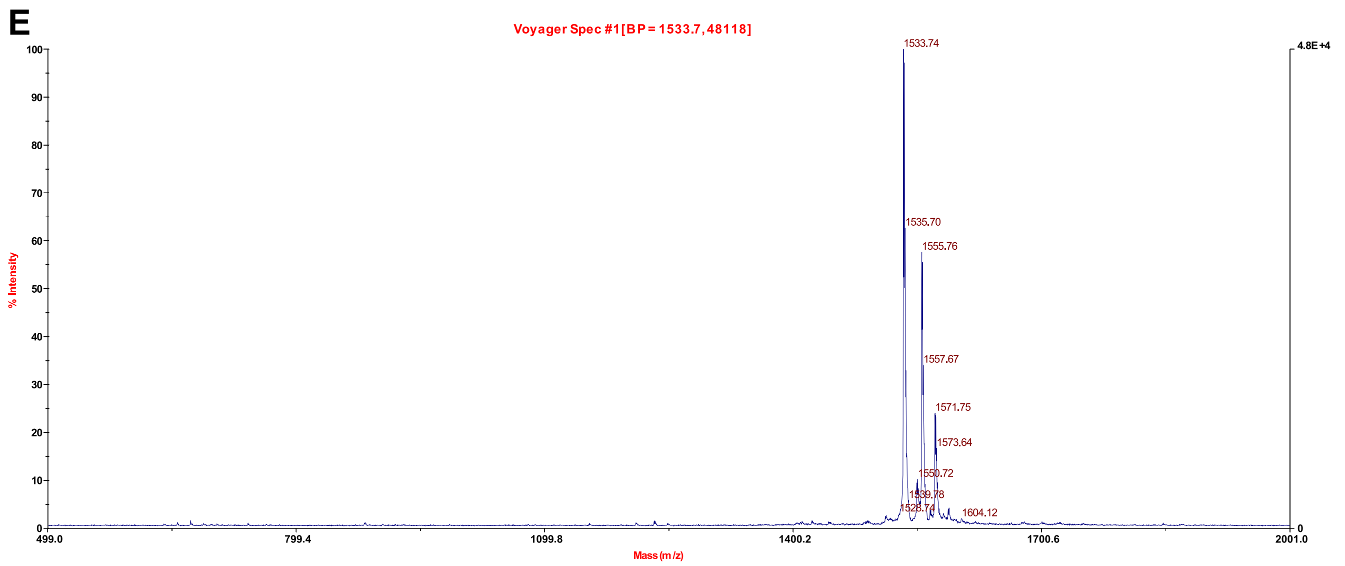


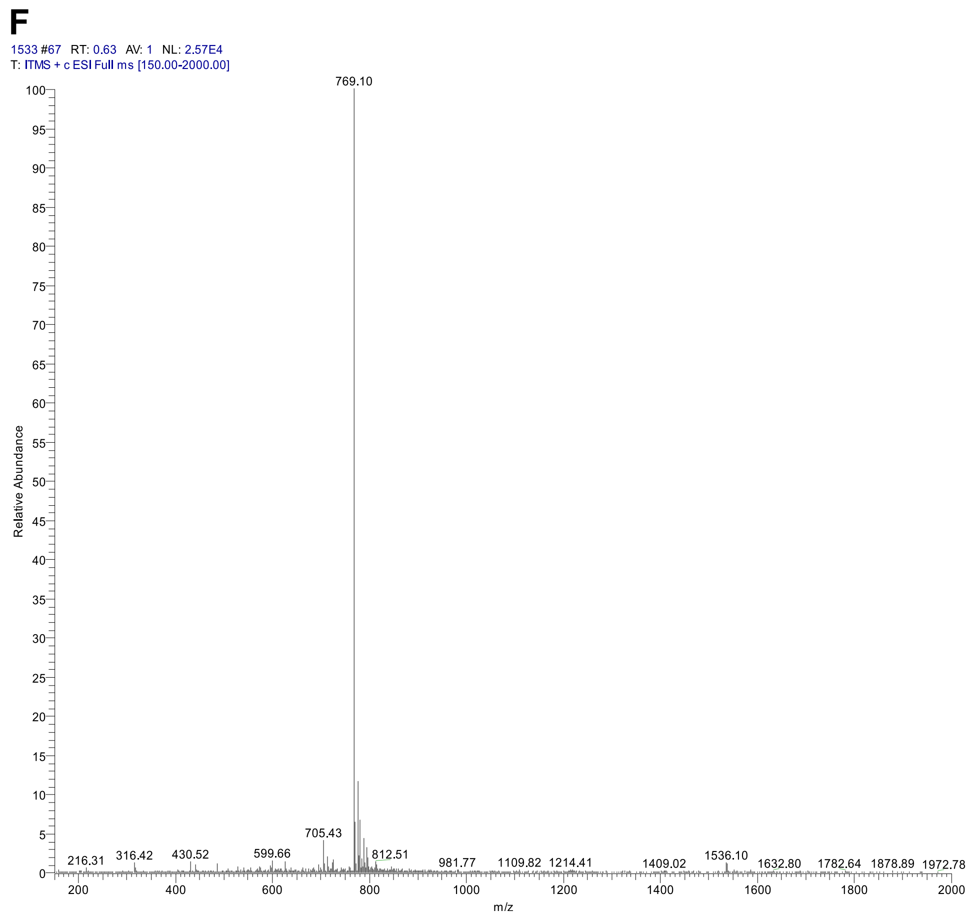


**Fig. 7.** (*continued*).

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**Table 2**
Minimum inhibitory concentrations of Alyteserins isolated from skin secretions of *A. maurus* against different microorganisms. MIC values are given in μM.

| | Net charge | *E. coli* | *S. aureus* | *C. albicans* |
|---|---|---|---|---|
| Alyteserin-1Ma | 2.1 | 214 | 214 | >400 |
| Alyteserin-1Mb | 3.1 | 217 | 434 | >400 |
| Alyteserin-2Ma | 2.1 | 38.4 | 9.5 | 150 |
| Alyteserin-2Mb | 1.1 | >400 | 19.3 | 310 |

Emonds, 2011; Vanhoye et al., 2003) despite the often high divergence between the AMP precursors of some constituent families (e.g., Hylidae and Ranidae). The differences between these two systems with respect to the gene architecture and otherwise highly conserved signal sequences provide further evidence for their convergent evolution (König and Bininda-Emonds, 2011). Also of note are the different cleavage sites for post-translational processing between the groups, where neobatrachian frogs commonly use a dibasic cleavage site (-RR- or -KR-) instead of the N-terminal -REIR- motif. Confusing the issue here, admittedly, is that the latter site is also used for *Xenopus* skin peptide precursors (Gibson et al., 1986), despite *Xenopus* (Pipidae) and Costata not forming a clade according to present opinion. However, we can only speculate about the impact of such modifications in the biosynthetic pathway of the precursor given that our knowledge about the endoproteolytic enzymes involved in this process comes exclusively from *X. laevis* (Kuks et al., 1989), while it remains obscure for all other anuran taxa.

Yet, despite their apparent informativeness as taxonomic indicators in evolutionary studies, biochemical data from frog skin secretions are rarely included in this context. Nevertheless, the presence of certain peptide families in the skins of two different frog species can serve as a useful tool to support a close evolutionary relationship between the species, distinguish populations (Conlon et al., 2010, 2004, 2011; Mechkarska et al., 2011) or, in combination with molecular data from mitochondrial genes, even lead to rejection of subspecies status (Conlon et al., 2006). Above the active peptides, information about the expressed host-defence peptide genes themselves (i.e., the architecture of a gene or the presence or absence of tandem repeats) enables statements about the gene (sub-) families to which they belong and thus can support evolutionary relationships between more inclusive taxa.

### *4.2. Is there a different selective pressure behind the origin of the anuran AMP system?*

Generally speaking, when present, neuroactive peptides (e.g., BLPs) and AMPs co-occur in the skin secretions of frog species (Fig. 1) and evidently fulfil defensive functions against either macro- (vertebrates) or micropredators (microorganisms). However, important differences between neuroactive peptides and AMPs are apparent.

Neuroactive skin peptides of the same class appear to be relatively well-conserved across anuran amphibians in terms of their primary structure. This phenomenon applies both for BLPs (as indicated in part by our results), but also for the bradykinin-related peptides [unpublished observations: König, Clark, Shaw]. Amino acid substitutions do occasionally occur within neuropeptide homologues, but with a distinct bias to particular sites that are apparently less important for receptor binding, thereby reflecting neutral drift. Thus, the evolutionary conserved regions observed in frog skin neuropeptides can be regarded as a result of an adaptation process to repel those predators predominant in the frog's habitat given that they strike against the species-specific receptor in a ligand-mimicking manner. The selective pressure thus brought about by the predators can therefore also lead to cases of convergent evolution of the same venomous compound, as was recently shown for caeruleins in the distantly related frogs *Litoria splendida* and *Lithobates catesbeianus* (Roelants et al., 2010).

By contrast, AMPs between even two closely related species tend to be highly different and the distributions of the different AMP families (e.g., bombinin or alyteserin) are highly restricted taxonomically. What binds AMPs as a group together is their common, cytolytic mechanism of action (Matsuzaki, 1999) that is more reliant upon the biophysical properties of the peptide (e.g., hydrophobicity and formation of helices in a lipoid environment) rather than its primary structure *per* se. Nevertheless, the amino acid sequence does have an impact on the antimicrobial potency of the peptide. For example, alyteserin-1a, -1b, and -1c from *A. obstetricians* (Conlon et al., 2009a) are ten times more potent against the Gram-negative *E. coli* than alyteserin-1Ma and -1Mb isolated in our study.

Although, the definite effect of single amino acid substitutions is not completely understood, the membrane constitution of the target cell (Zasloff, 2002) should not be under-estimated in this regard. Thus, the antimicrobial activity of AMPs probably cannot be regarded as a result of specificity against prokaryotic cells, although they appear to have increased effectivity against them because of the differences in hydrophobic and electrostatic interactions between AMPs and either eukaryotic or prokaryotic cell membranes. For example, prokaryotic membranes are more prone to disruption by AMPs due to the greater abundance of anionic lipids (e.g., phospholipids) in the outer leaflets of their bilayers (Zasloff, 2002). However, a considerable number of anuran AMPs also act on eukaryotic cells and can inhibit the growth of fungi and yeast (e.g., *C. albicans*) or exhibit haemolytic activity at high concentrations. These characteristics potentially make AMPs toxic against macropredators as well and deserve an important consideration with respect to the development of new therapeutic drugs. The recognition of AMPs as general cytolysins has to be stressed because this class of peptide has been long considered to be part of the anuran innate immune system (Conlon, 2010; Nicolas and Mor, 1995; Woodhams et al., 2007). Although AMPs unquestionably do present an adaptive benefit with respect to

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antimicrobial immunity when they are present in a species, we hypothesise that the original function of AMPs was not exclusively antimicrobial and that this latter function instead may actually have evolved secondarily as a by-product of glandular metabolism incorporated for defensive purposes into the frog skin. Accordingly, our hypothesis potentially explains the apparent lack of AMPs in many frog species as well as the apparent convergent nature of frog skin peptide genes.

## 5. Conclusion

Amphibian skin provides by far the richest source of novel host-defence peptides so far found within the Animal Kingdom. Using molecular cloning techniques, we isolated four novel AMPs as well as the neuroactive peptide alytesin from the skin secretions of *A. maurus*. Importantly, this method enabled us to provide the first genetic data for the genes underlying these peptides for any member of Alytidae. Altogether, these data reveal a close similarity between the AMP systems for Alytidae and Bombinatoridae, providing independent molecular support for their inclusion in the monophyletic clade Costata. At the same time, the Costata AMP system shows important differences with that of Neobatrachia, strengthening the hypothesis that they have evolved independently of one another. On the basis of our results, we present arguments that the AMP system in anuran amphibians did not evolve initially as part of the innate immune system of these animals as is commonly thought, but instead forms part of a general chemical defensive strategy as cytolysins that were secondarily co-opted to assist antimicrobial immunity.

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## Conflict of interest

We declare that there is no conflict of interest among the authors.

## References

Ali, M.F., Soto, A., Knoop, F.C., Conlon, J.M., 2001. Antimicrobial peptides isolated from skin secretions of the diploid frog, *Xenopus tropicalis* (Pipidae). Biochim. Biophys. Acta Protein Struct. Mol. Enzymol. 1550, 81–89.

Amiche, M., Ladram, A., Nicolas, P., 2008. A consistent nomenclature of antimicrobial peptides isolated from frogs of the subfamily Phyllomedusinae. Peptides 29, 2074–2082.

Anastasi, A., 1971. Alytesin and bombesin, two peptide analogs from amphibian skin. Naunyn Schmiedebergs Arch. Pharmacol. 269, 135–139.

Anastasi, A., Erspamer, V., Endean, R., 1975. Amino acid composition and sequence of litorin, a bombesin-like nonapeptide from the skin of the Australian leptodactylid frog *Litoria aurea*. Experientia 31, 510–511.

Barra, D., Erspamer, G.F., Simmaco, M., Bossa, F., Melchiorri, P., Erspamer, V., 1985. Rohdei-litorin – a new peptide from the skin of *Phyllomedusa rohdei*. FEBS Lett. 182, 53–56.

Bevins, C.L., Zasloff, M., 1990. Peptides from frog-skin. Annu. Rev. Biochem. 59, 395–414.

Calderon, L.D., Silva, A.D.E., Ciancaglini, P., Stabeli, R.G., 2011. Antimicrobial peptides from *Phyllomedusa* frogs: from biomolecular diversity to potential nanotechnologic medical applications. Amino Acids 40, 29–49.

Clarke, B.T., 1997. The natural history of amphibian skin secretions, their normal functioning and potential medical applications. Biol. Rev. 72, 365–379.

Conlon, J.M., 2008a. A proposed nomenclature for antimicrobial peptides from frogs of the genus *Leptodactylus*. Peptides 29, 1631–1632.

Conlon, J.M., 2008b. Reflections on a systematic nomenclature for antimicrobial peptides from the skins of frogs of the family Ranidae. Peptides 29, 1815–1819.

Conlon, J.M., 2010. The contribution of skin antimicrobial peptides to the system of innate immunity in anurans. Cell Tissue Res. 343, 201–212.

Conlon, J.M., Al-Ghafari, N., Coquet, L., Leprince, J., Jouenne, T., Vaudry, H., Davidson, C., 2006. Evidence from peptidomic analysis of skin secretions that the red-legged frogs, *Rana aurora draytonii* and *Rana aurora aurora*, are distinct species. Peptides 27, 1305–1312.

Conlon, J.M., Coquet, L., Leprince, J., Jouenne, T., Vaudry, H., King, J.D., 2010. Primary structures of skin antimicrobial peptides indicate a close, but not conspecific, phylogenetic relationship between the leopard frogs *Lithobates onca* and *Lithobates yavapaiensis* (Ranidae). Comp. Biochem. Phys. C 151, 313–317.

Conlon, J.M., Demandt, A., Nielsen, P.F., Leprince, J., Vaudry, H., Woodhams, D.C., 2009a. The alyteserins: two families of antimicrobial peptides from the skin secretions of the midwife toad *Alytes obstetricans* (Alytidae). Peptides 30, 1069–1073.

Conlon, J.M., Iwamuro, S., King, J.D., 2009b. Dermal cytolytic peptides and the system of innate immunity in anurans. Ann. N. Y. Acad. Sci. 1163, 75–82.

Conlon, J.M., Kolodziejek, J., Nowotny, N., 2004. Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. Biochim. Biophys. Acta 1696, 1–14.

Conlon, J.M., Mechkarska, M., Coquet, L., Jouenne, T., Leprince, J., Vaudry, H., Kolodziejek, J., Nowotny, N., King, J.D., 2011. Characterization of antimicrobial peptides in skin secretions from discrete populations of *Lithobates chiricahuensis* (Ranidae) from central and southern Arizona. Peptides 32, 664–669.

Daly, J.W., Spande, T.F., Garraffo, H.M., 2005. Alkaloids from amphibian skin: a tabulation of over eight-hundred compounds. J. Nat. Prod. 68, 1556–1575.

Duda, T.F., Vanhoye, D., Nicolas, P., 2002. Roles of diversifying selection and coordinated evolution in the evolution of amphibian antimicrobial peptides. Mol. Biol. Evol. 19, 858–864.

Erspamer, V., 1994. Bioactive secretions of the amphibian integument. In: Heatwole, H., Barthalmus, G.T. (Eds.), Amphibian Biology. The Integument, vol. 1. Surrey Beatty and Sons, Chipping Norton, pp. 178–350.

Fleury, Y., Vouille, V., Beven, L., Amiche, M., Wroblewski, H., Delfour, A., Nicolas, P., 1998. Synthesis, antimicrobial activity and gene structure of a novel member of the dermaseptin B family. Biochim. Biophys. Acta Gene Struct. Expr. 1396, 228–236.

Frost, D.R., 2011. Amphibian Species of the World: An Online Reference. Version 5.5 (31 January, 2011). American Museum of Natural History, New York, USA. Electronic Database accessible at: http://research.amnh.org/vz/herpetology/amphibia/,.

Frost, D.R., Grant, T., Faivovich, J., Bain, R.H., Haas, A., Haddad, C.F.B., De Sa, R.O., Channing, A., Wilkinson, M., Donnellan, S.C., Raxworthy, C.J., Campbell, J.A., Blotto, B.L., Moler, P., Drewes, R.C., Nussbaum, R.A., Lynch, J.D., Green, D.M., Wheeler, W.C., 2006. The amphibian tree of life. Bull. Am. Mus. Nat. Hist., 8–371.

Gibson, B.W., Poulter, L., Williams, D.H., Maggio, J.E., 1986. Novel peptide-fragments originating from PGLa and the Caerulein and Xenopsin precursors from *Xenopus laevis*. J. Biol. Chem. 261, 5341–5349.

Haas, A., 2003. Phylogeny of frogs as inferred from primarily larval characters (Amphibia: Anura). Cladistics 19, 23–89.

König, E., Bininda-Emonds, O.R.P., 2011. Evidence for convergent evolution in the antimicrobial peptide system in anuran amphibians. Peptides 32, 20–25.

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Kuks, P.F.M., Creminon, C., Leseney, A.M., Bourdais, J., Morel, A., Cohen, P., 1989. *Xenopus laevis* skin Arg-Xaa-Val-Arg-Gly-endoprotease – a highly specific protease cleaving after a single arginine of a consensus sequence of peptide-hormone precursors. J. Biol. Chem. 264, 14609–14612.

Lazarus, L.H., Attila, M., 1993. The toad, ugly and venomous, wears yet a precious jewel in his skin. Prog. Neurobiol. 41, 473–507.

Martínez-Solano, I., Gonçalves, H.A., Arntzen, J.W., García-París, M., 2004. Phylogenetic relationships and biogeography of midwife toads (Discoglossidae: *Alytes*). J. Biogeogr. 31, 603–618.

Matsuzaki, K., 1999. Why and how are peptide–lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. Biochim. Biophys. Acta Biomembr. 1462, 1–10.

Mechkarska, M., Ahmed, E., Coquet, L., Leprince, J., Jouenne, T., Vaudry, H., King, J.D., Conlon, J.M., 2011. Peptidomic analysis of skin secretions demonstrates that the allopatric populations of *Xenopus muelleri* (Pipidae) are not conspecific. Peptides 32, 1502–1508.

Miele, R., Ponti, D., Boman, H.G., Barra, D., Simmaco, M., 1998. Molecular cloning of a bombinin gene from *Bombina orientalis*: detection of NF-[kappa]B and NF-IL6 binding sites in its promoter. FEBS Lett. 431, 23–28.

Nagalla, S.R., Gibson, B.W., Tang, D.Z., Reeve, J.R., Spindel, E.R., 1992. Gastrin-releasing peptide (GRP) is not mammalian bombesin – identification and molecular-cloning of a true amphibian GRP distinct from amphibian bombesin in *Bombina orientalis*. J. Biol. Chem. 267, 6916–6922.

Nakajima, T., Tanimura, T., Pisano, J.J., 1970. Isolation and structure of a new vasoactive polypeptide. Fed. Proc. 29, 282.

Nicolas, P., Mor, A., 1995. Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Annu. Rev. Microbiol. 49, 277–304.

Pukala, T.L., Bowie, J.H., Maselli, V.M., Musgrave, I.F., Tyler, M.J., 2006. Host-defence peptides from the glandular secretions of amphibians: structure and activity. Nat. Prod. Rep. 23, 368–393.

Roelants, K., Fry, B.G., Norman, J.A., Clynen, E., Schoofs, L., Bossuyt, F., 2010. Identical skin toxins by convergent molecular adaptation in frogs. Curr. Biol. 20, 125–130.

Roelants, K., Gower, D.J., Wilkinson, M., Loader, S.P., Biju, S.D., Guillaume, K., Moriau, L., Bossuyt, F., 2007. Global patterns of diversification in the history of modern amphibians. Proc. Natl. Acad. Sci. U. S. A. 104, 887–892.

Simmaco, M., Kreil, G., Barra, D., 2009. Bombinins, antimicrobial peptides from Bombina species. Biochim. Biophys. Acta Biomembr. 1788, 1551–1555.

Spindel, E.R., 2006. Amphibian bombesin-like peptides. In: Kastin, J.A. (Ed.), Handbook of Biologically Active Peptides, first ed. Academic Press, Amsterdam, pp. 277–281.

Tennessen, J.A., 2005. Molecular evolution of animal antimicrobial peptides: widespread moderate positive selection. J. Evol. Biol. 18, 1387–1394.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Tyler, M.J., Stone, D.J.M., Bowie, J.H., 1992. A novel method for the release and collection of dermal, glandular secretions from the skin of frogs. J. Pharmacol. Toxicol. 28, 199–200.

Vanhoye, D., Bruston, F., Nicolas, P., Amiche, M., 2003. Antimicrobial peptides from hylid and ranin frogs originated from a 150-million-year-old ancestral precursor with a conserved signal peptide but a hypermutable antimicrobial domain. Eur. J. Biochem. 270, 2068–2081.

Vouille, V., Amiche, M., Nicolas, P., 1997. Structure of genes for dermaseptins B, antimicrobial peptides from frog skin – exon 1-encoded prepropeptide is conserved in genes for peptides of highly different structures and activities. FEBS Lett. 414, 27–32.

Wang, L., Zhou, M., Lynch, L., Chen, T.B., Walker, B., Shaw, C., 2009. Kassina senegalensis skin tachykinins: molecular cloning of kassinin and (Thr(2), Ile(9))-kassinin biosynthetic precursor cDNAs and comparative bioactivity of mature tachykinins on the smooth muscle of rat urinary bladder. Biochimie 91, 613–619.

Wang, T., Zhang, J., Shen, J.H., Jin, Y., Lee, W.H., Zhang, Y., 2005. Maximins S, a novel group of antimicrobial peptides from toad *Bombina maxima*. Biochem. Biophys. Res. Commun. 327, 945–951.

Woodhams, D.C., Rollins-Smith, L.A., Alford, R.A., Simon, M.A., Harris, R.N., 2007. Innate immune defenses of amphibian skin: antimicrobial peptides and more. Anim. Conservation 10, 425–428.

Yasuhara, T., Hira, M., Nakajima, T., Yanaihar, N., Yanaihar, C., Hashimot, T., Sakura, N., Tachiban, S., Araki, K., Bessho, M., Yamanaka, T., 1973. Active peptides on smooth-muscle in skin of *Bombina orientalis* Boulenger and characterization of a new bradykinin analogue. Chem. Pharm. Bull. 21, 1388–1391.

Yasuhara, T., Nakajima, T., Nokihara, K., Yanaihara, C., Yanaihara, N., Erspamer, V., Erspamer, G.F., 1983. Two new frog skin peptides, phyllolitorins, of the bombesin–ranatensin family from *Phyllomedusa sauvagei*. Biomed. Res. 4, 407–411.

Zasloff, M., 2002. Antimicrobial peptides of multicellular organisms. Nature 415, 389–395.