available at www.sciencedirect.com
SCIENCE DIRECT®
journal homepage: www.elsevier.com/locate/peptides

# Pelophylaxins: Novel antimicrobial peptide homologs from the skin secretion of the Fukien gold-striped pond frog, *Pelophylax plancyi fukienensis* Identification by "shotgun" cDNA cloning and sequence analysis

*Mei Zhou, Tianbao Chen, Brian Walker, Chris Shaw* *

*School of Pharmacy, Queen's University, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland, UK*

ARTICLE INFO

*Article history:*
Received 9 June 2005
Received in revised form
20 July 2005
Accepted 20 July 2005

*Keywords:*
Amphibian
Venom
Mass spectrometry
Peptide
Cloning

ABSTRACT

Amphibian skin secretions are rich in antimicrobial peptides that act as important components of an innate immune system. Here, we describe a novel "shotgun" skin peptide precursor cloning technique that facilitates rapid access to these genetically encoded molecules and effects their subsequent identification and structural characterization from the secretory peptidome. Adopting this approach on a skin secretion-derived library from a hitherto unstudied Chinese species of frog, we identified a family of novel antimicrobial peptide homologs, named pelophylaxins, that belong to previously identified families (ranatuerins, brevinins and temporins) found predominantly in the skin secretions from frogs of the genus *Rana*. These data further substantiate the scientifically robust nature of applying parallel transcriptome and peptidome analyses on frog defensive skin secretions that can be obtained in a non-invasive, non-destructive manner. In addition, the present data illustrate that rapid structural characterization of frog skin secretion peptides can be achieved from an unstudied species without prior knowledge of primary structures of endogenous peptides.



## 1. Introduction

Amphibian skin is, in terms of morphology, biochemistry and physiology, a complicated organ that performs a plethora of functions dedicated to the survival of the organism in what are often extreme environmental conditions [1,6,10]. The complex cocktails of bioactive molecules contained in amphibian skin secretions appear to have evolved for passive chemical defense against predators [1,6,10]. The source of these biologically active compounds are the dermal granular glands and biochemically, the constituent molecules are representative of many classes including biogenic amines, peptides, proteins, alkaloids and heterocyclics [1,6,10]. As a consequence, extracts of amphibian skin have been used for centuries in folk medicine and witchcraft due to their possession of a wide spectrum of pharmacological effects [9,10]. The structural diversity of peptides in these amphibian defensive skin secretions probably reflects a broad spectrum of biological roles [1,6,10].

In recent years, the emergence of multiple drug-resistant pathogenic microorganisms has stimulated the search for new, naturally occurring or synthetic bactericides and fungicides that may have therapeutic value [13]. Polypeptides

* *Corresponding author*. Tel.: +44 2890 972129; fax: +44 2890 247794.
*E-mail address:* chris.shaw@qub.ac.uk (C. Shaw).
0196-9781/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2005.07.007

with broad spectrum anti-microbial activity, synthesized in amphibian skin granular glands, have been the subject of increasing attention and appraisal as potential novel therapeutics. However, as this is happening, global amphibian populations are in rapid decline. In some instances, declines have been gradual, but in others, they have been dramatic and short term, even extending to extinction events as exemplified by the Costa Rican golden toad (*Bufo periglenes*) and the Australian gastric brooding frogs (*Rheobatrachus* spp.) [2,7].

Here, we describe the application and integration of several non-invasive sampling techniques that can effect rapid identification and structural characterization of amphibian skin secretion peptides from unstudied species in the absence of any primary structural data on selected secretion peptides. By using a degenerate primer designed to a consensus nucleotide sequence in the 5′-untranslated region of previously characterized frog skin peptide cDNAs and in a 3′-RACE reaction on a cDNA library constructed from lyophilized skin secretion, we were able to identify a series of novel peptides from the unstudied Chinese frog, *Pelophylax plancyi fukienensis* (the Fukien gold-striped pond frog). The NCBI-based BLAST search found that all of the cDNA-deduced and subsequently identified peptides could be identified as structural homologs of previously identified antimicrobial peptide families from frog species of the genus *Rana* [5,15,17]. In keeping with established nomenclature, the antimicrobial peptide homologs identified and characterized in the present study were named pelophylaxins, and the nucleotide structures of their cloned cDNAs have been deposited in the public EMBL database.

## 2. Materials and methods

### 2.1. *Specimen biodata and secretion harvesting*

Adult specimens of the Fukien gold-striped pond frog, *P. plancyi fukienensis* ($n = 3$, snout-to-vent length 7 cm) were captured in paddy fields around Fuzhou City, Fujian Province, People's Republic of China (Fukien is the old name for Fujian). Secretion harvesting was performed in the field after which the frogs were released unharmed. Skin secretion was obtained from the dorsal skin using gentle transdermal electrical stimulation as previously described [16]. The stimulated secretions were washed from the skin using deionized water and divided into either 0.2% (v/v) aqueous trifluoroacetic acid (for subsequent peptide characterization) or into cell lysis/mRNA stabilization buffer (Dynal) for subsequent cDNA library construction.

### 2.2. *"Shotgun" cloning of prepropelophylaxin cDNAs*

Polyadenylated mRNA was isolated from the stabilization buffer/skin secretion mixture using magnetic oligo-dT beads as described by the manufacturer (Dynal Biotech, UK) and reverse-transcribed. The cDNA was subjected to 3′-RACE procedures to obtain full-length prepropelophylaxin nucleic acid sequence data using a SMART-RACE kit (Clontech UK) essentially as described by the manufacturer. Briefly, the 3′-RACE reactions employed a NUP primer (supplied with the kit) and a degenerate sense primer (S1; 5′-GAWYYAYYHRAGC-CYAAADATG-3′) that was designed for a highly conserved domain of the 5′-untranslated region of previously characterized antimicrobial peptide cDNAs from *Rana* frog species [5,15,17]. The PCR cycling procedure was as follows: initial denaturation step: 60 s at 94 °C; 35 cycles: denaturation 30 s at 94 °C, primer annealing for 30 s at 53 °C; extension for 180 s at 72 °C. PCR products were gel-purified, cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated sequencer.

### 2.3. *Identification and structural analyses of cloned cDNA-deduced pelophylaxins*

The subsequently acidified skin secretion washings were clarified of microparticulates by centrifugation (13,000 × *g* for 10 min). The decanted clear supernatant was then pumped directly onto a reverse phase HPLC column and subjected to LC/MS using a gradient formed from 0.05/99.5 (v/v) TFA/water to 0.05/19.95/80.0 (v/v/v) TFA/water/acetonitrile in 240 min at a flow rate of 1 ml/min. A thermoquest gradient reversed phase HPLC system, fitted with an analytical column (C-18) and interfaced with a Thermoquest LCQ$^{TM}$ Deca electrospray ion-trap mass spectrometer, was employed. The effluent from the chromatographic column was flow-split with approximately 10% entering the mass spectrometer source and 90% directed towards a fraction collector. Dead volume between column and fraction collector was minimal (20 μl). The molecular masses of polypeptides in each chromatographic fraction were further analyzed using matrix-assisted laser desorption/ionization, time-of-flight-mass spectrometry (MALDI-TOF-MS) on a linear time-of-flight Voyager DE PRO mass spectrometer (Perseptive Biosystems, MA, USA) in positive detection mode using alpha-cyano-4-hydroxycinnamic acid as the matrix. Internal mass calibration of the instrument with known standards established the accuracy of mass determination as ±0.1%. The peptides with masses coincident with those of pelophylaxins as deduced from the cloned precursor cDNAs were each subjected to primary structural analyses by automated Edman degradation using an Applied Biosystems 491 Procise sequencer in pulsed-liquid mode or by MS/MS fragmentation sequencing using the LCQ$^{TM}$ Deca.

## 3. Results

### 3.1. *"Shotgun" cloning of prepropelophylaxin cDNAs*

Four novel peptide-encoding cDNAs, each containing the deduced primary structure of novel peptides that were named pelophylaxins 1 through 4, respectively, were consistently cloned from the skin secretion library (Fig. 1). An NCBI-BLAST search found that the nucleotide sequences of the four novel prepropelophylaxin open-reading frames, exhibited between 75 and 95% sequence identity with the corresponding antimicrobial peptides from *Rana* frogs. The conserved preproregion of each peptide precursor open-reading frame includes a putative 22 amino acid residue signal peptide followed by an acidic peptide that terminates in a typical -Lys-Arg- (-KR-) propeptide convertase processing motif that is

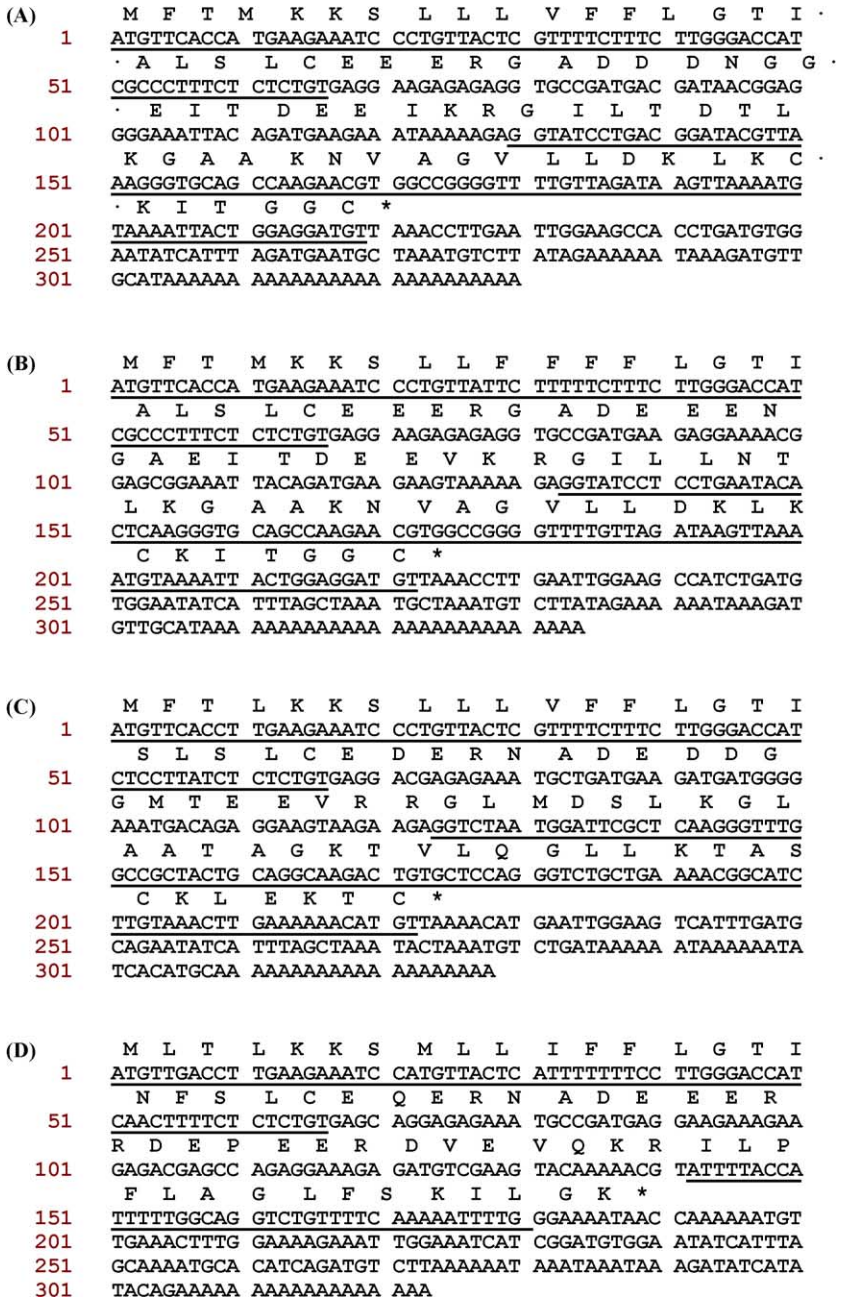


**Fig. 1 – Nucleotide sequences of cDNAs cloned from a skin secretion library of the Fukien gold-striped pond frog (*P. plancyi fukienensis*) that encode pelophylaxins 1 through 4 (A–D), respectively. Putative signal peptides are double-underlined, mature peptides as identified in skin secretion are single-underlined and stop codons are indicated by asterisks.**

responsible for cleavage and release of each respective mature peptide located at the C-terminals (Fig. 2).

### 3.2. *Identification and structural characterization of pelophylaxins*

Following the prediction of the molecular masses of the four novel peptides (pelophylaxins) from the cloned precursors and compensation for post-translational modification (single disulfide bridge formation in the C-terminal loop = −2 amu), each mature peptide was identified in respective skin secretion HPLC fractions (Fig. 3 and Table 1). Primary structures were confirmed by MS/MS fragmentation (data not shown). The NCBI-BLAST search revealed that pelophylaxin-1 and -2 were most closely structurally related to ranatuerin 2C from the North American frogs *Rana clamitans*, *Rana pipiens*, *Rana palustris* and *Rana luteiventris* [5]. Pelophylaxin-3 was most closely structurally related to brevinin-1 from the Asiatic frog *Rana brevipoda porsa* and the European frog *Rana esculenta* [5]. Pelophylaxin-4 was most closely structurally related to temporin 1Cd from the North American frog *R. clamitans* [5]. The pelophylaxins thus represent the structural homologs of three established families of antimicrobial peptides from *Rana* frogs [5,17].

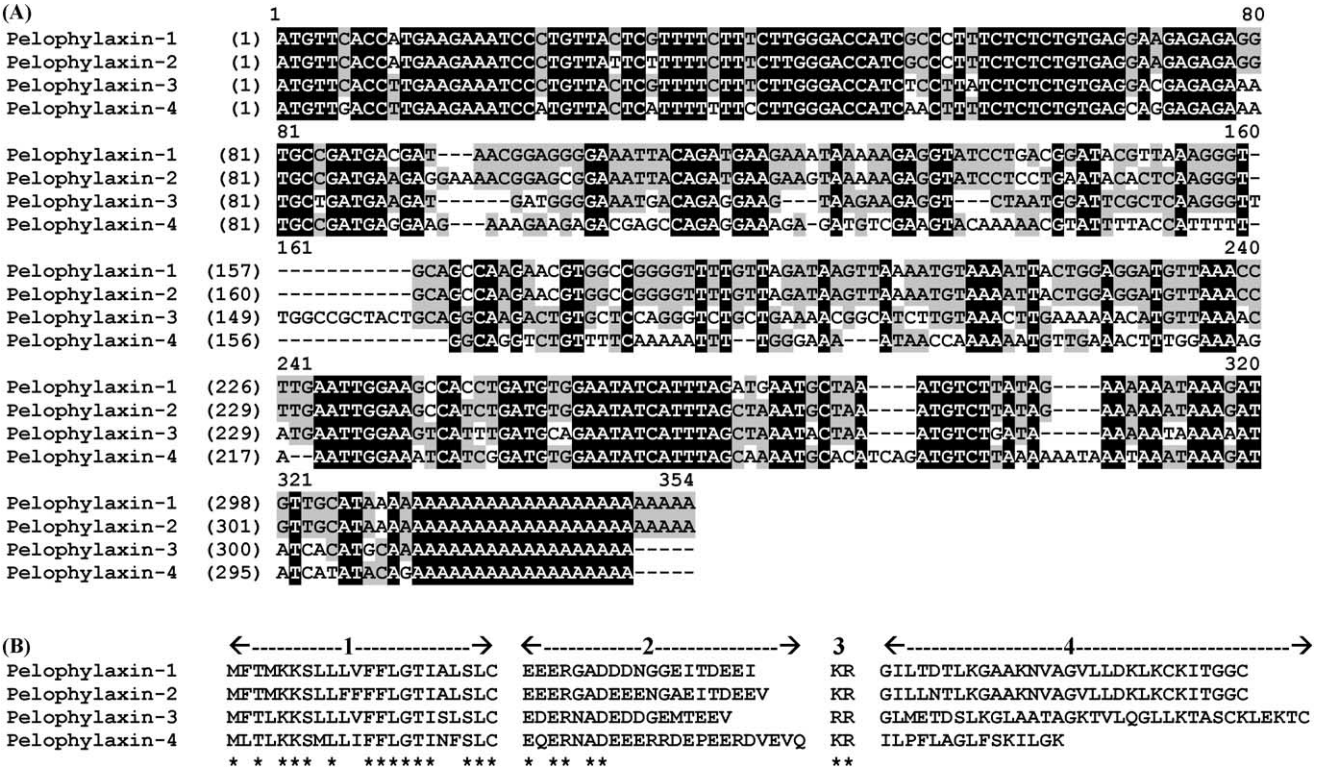


**Fig. 2 – (A) Alignment of nucleotide sequences of cDNA clones encoding pelophylaxins 1 through 4, respectively. Conserved nucleotides shown white on black and consensus nucleotides shown black on gray. Gaps have been included to maximize alignment. Note the highest degree of conserved nucleotides in the proximal, putative signal peptide-encoding domain. (B) Alignment of translated open-reading frames of pelophylaxins 1 through 4 indicating sequential domains: (1) putative signal peptide; (2) acidic "spacer" peptide; (3) dibasic residue propeptide convertase cleavage site; (4) variable bioactive peptide-encoding domain. Identical residues indicated by asterisks—note the majority reside within the signal peptide domain.**

## 4. Discussion

During the past decade, a large number of antimicrobial peptides from different organisms, including mammals, plants, arthropods, molluscs and bacteria, have been isolated and characterized. The defensive skin secretions of frogs are known to contain a plethora of biologically active peptides, some of which share common primary structural features with endogenous vertebrate regulatory peptides and others that appear to have no obvious structural counterparts in higher vertebrates,

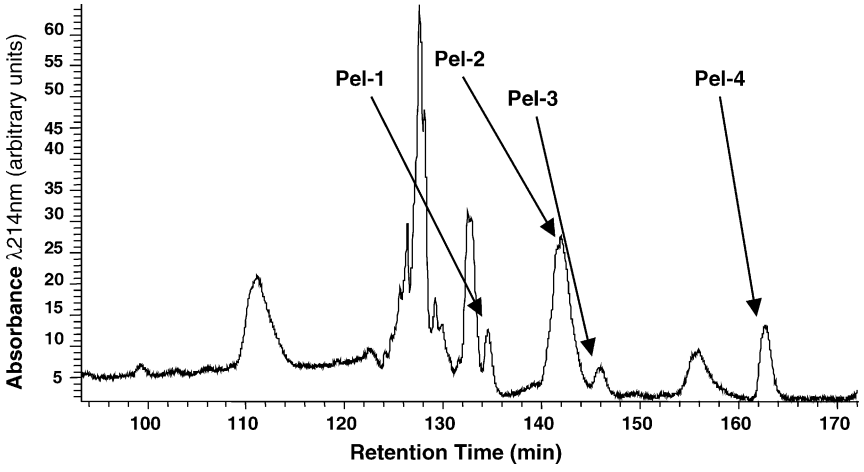


**Fig. 3 – Region of reverse phase HPLC chromatogram of *P. plancyi fukienensis* skin secretion indicating the absorbance peaks corresponding to pelophylaxins 1 through 4 (Pel-1 to Pel-4).**

**Table 1 – Molecular masses and primary structures of four novel peptides (pelophylaxins) identified in semi-preparative reverse phase HPLC fractions of Fukien gold-striped frog, *Pelophylax plancyi fukienensis*, skin secretion**

| Peptide | Original fraction | Mass observed (Da) | Mass calculated (Da) | Amino acid sequence |
|---|---|---|---|---|
| Pelophylaxin-1 | 136 | 2999.24 | 2998.61 | GILTDTLKGAAKNVAGVLLDKLKCKITGGC |
| Pelophylaxin-2 | 142 | 3010.85 | 3009.68 | GILLNTLKGAAKNVAGVLLDKLKCKITGGC |
| Pelophylaxin-3 | 146 | 3349.05 | 3348.01 | GLMDSLKGLAATAGKTVLQGLLKTASCKLEKTC |
| Pelophylaxin-4 | 163 | 1431.01 | 1430.82 | ILPFLAGLFSKIL–$NH_2$ |

for example, the amphipathic, cationic antimicrobial peptides [10,14]. These latter molecules represent the focus of a major research effort and this has yielded a rich variety of generally broad-spectrum antimicrobial peptides [10,14]. These peptides are released from specialized skin glands by stress-inducing stimuli to provide an effective and rapidly acting defense against potentially harmful microorganisms [10].

The rapid decline in amphibian populations throughout the world is alarming and some entire populations have completely disappeared in a relatively shorter period of time [8]. The scientific evaluation of the potential therapeutic lead compounds present in their defensive skin secretions is thus time-limited in the extreme and the rapid deployment of non-invasive, high-throughput analytical strategies is central to achieve acquisition of the maximum amount of data in the shortest possible time frames. Until recently, while isolation and structural characterization of proteins/peptides could be achieved using stored lyophilized skin secretions, construction of cDNA libraries for the purpose of molecular cloning of precursors necessitated sacrifice of the living specimen followed by removal of skin. These procedures thus made it extremely difficult to perform both aspects of the study on the same specimens and made it impossible to perform sequential experiments in time. By screening of cDNA clones, the existence of novel peptides can be predicted from the amino acid sequence of precursors [12]. A novel method recently developed in our laboratory permits both isolation and sequencing of peptides and cloning of homologous cDNAs from the same single samples of lyophilized secretions [3,4]. It is reiterated here that the sacrifice of frogs for skin cDNA library construction, hitherto regarded as a *sine qua non* for the cloning of granular gland secretion components, is unnecessary and that robustness of appropriate molecular genetic data is not compromised.

Four novel antimicrobial peptide homologs, named pelophylaxins 1 through 4, have been identified in the defensive skin secretion of the hitherto unstudied Chinese frog, *P. plancyi fukienensis*, by initial ''shotgun'' cloning of precursor-encoding cDNAs from a skin secretion-derived library and subsequently by identification in reverse phase HPLC fractions of skin secretion followed by structural characterization. The structural organization of prepropelophylaxins, deduced from respective cloned cDNAs, is highly conserved with respect to other frog skin antimicrobial peptide precursors warehoused in public databases. The topographical organization consists of a putative signal peptide (approximately 22 residues), a ''spacer'' peptide of variable size that is rich in acidic amino acid residues, a single paired basic amino acid residue propeptide convertase processing site and a single copy of antimicrobial peptide located at the C-terminus. These extensive and highly conserved similarities of gene transcript organization support the hypothesis that the antimicrobial genes might come from a common ancestor by duplication events [11].

It is worth noting that all amphibian skin antimicrobial peptide precursors possess a signal peptide of 22 amino acid residues that terminates at a cysteinyl residue, the most likely cleavage site for the endoproteolytic ''signal peptidase''. The highly conserved acidic amino acid residue-rich ''spacer'' peptide located between the signal peptide and the variable bioactive peptide encoding domain is presumed to be of an as yet undetermined functional significance [11,17].

The presence of highly conserved nucleic acid domains within the transcripts encoding these peptide precursors is an attribute that has been used in the present study to design ''universal'' primers that can effect rapid molecular cloning of such precursors from unstudied species in a scientifically robust and valid manner. The discovery that the granular gland transcripts are present in stimulated defensive skin secretions [3,4] has permitted the parallel structural characterization of prepropeptides and their mature products in the same samples of secretion. These samples can be taken in a non-invasive manner even under field conditions—a factor that aids in the conservation of already endangered native amphibian populations.

## 5. Data deposition footnote

The nucleotide sequences of prepropelophylaxins, encoding respective pelophylaxins named 1 through 4, from the skin secretion of *P. plancyi fukienensis*, have been deposited in the EMBL Nucleotide Sequence Database under accession codes AJ972867 through AJ972870.

## Acknowledgement

Mei Zhou is in receipt of an Overseas Studentship at Queen's University, Belfast.

## REFERENCES

[1] Bevins CL, Zasloff M. Peptides from frog skin. Ann Rev Biochem 1990;59:395–414.
[2] Berger L, et al. Chytridiomycosis causes amphibian mortality associated with population declines in the rainforests of Australia and Central America. Proc Natl Acad Sci USA 1998;95:9031–6.
[3] Chen T, Farragher S, Bjourson AJ, Orr DF, Rao P, Shaw C. Granular gland transcriptomes in stimulated amphibian skin secretions. Biochem J 2003;371:125–30.

[4] Chen T, Bjourson AJ, McClean S, Orr DF, O'Kane EJ, Rao P, et al. Cloning of maximakinin precursor cDNAs from Chinese toad, *Bombina maxima*, venom. Peptides 2003;24:853–61.
[5] Conlon JM, Kolodziejek J, Nowotny N. Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. Biochim Biophys Acta 2004;1696:1–14.
[6] Erspamer V. Bioactive secretions of the integument. In: Heatwole H, Barthalmus GT, editors. Amphibian Biology, Vol. 1. The Integument. Chipping Norton: Surrey Beatty & Sons, 1994 [chapter 9].
[7] Foster P. The potential negative impacts of global climate change on tropical montane cloud forests. Earth Sci Rev 2001;55:73–106.
[8] Houlahan JE, Findlay CS, Schmidt BR, Meyer AH, Kuzmin SL. Quantitative evidence for global amphibian population declines. Nature 2000;404:752–5.
[9] Jacob L, Zasloff M. Potential therapeutic applications of magainins and other antimicrobial agents of animal origin. Ciba Found Symp 1994;186:197–216.
[10] Lazarus LH, Atilla M. The toad, ugly and venomous, wears yet a precious jewel in his skin. Prog Neurobiol 1993;41:473–507.
[11] Nicolas P, Mor A. Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Annu Rev Microbiol 1995;49:277–304.
[12] Richter K, Egger R, Kreil G. D-alanine in the frog skin peptide dermorphin is derived from L-alanine in the precursor. Science 1987;238:200–2.
[13] Rybak MJ. Resistance to antimicrobial agents: an update. Pharmacotherapy 2004;24:203–15.
[14] Simmaco M, Mignogna G, Barra D. Antimicrobial peptides from amphibian skin: what do they tell us? Biopolymers 1998;47:435–50.
[15] Simmaco M, Mignogna G, Barra D, Bossa F. Antimicrobial peptides from skin secretions of *Rana esculenta*. Molecular cloning of cDNAs encoding esculentin and brevinins and isolation of new active peptides. J Biol Chem 1994;269:11956–61.
[16] Tyler MJ, Stone DJM, Bowie JH. A novel method for the release and collection of dermal, glandular secretions from the skin of frogs. J Pharmacol Toxicol Meth 1992;28:199–200.
[17] Vanhoye D, Bruston F, Nicolas P, Amiche M. Antimicrobial peptides from hylid and ranid frogs originated from a 150-million-year-old ancestral precursor with a conserved signal peptide but a hypermutable antimicrobial domain. Eur J Biochem 2003;270:2068–81.