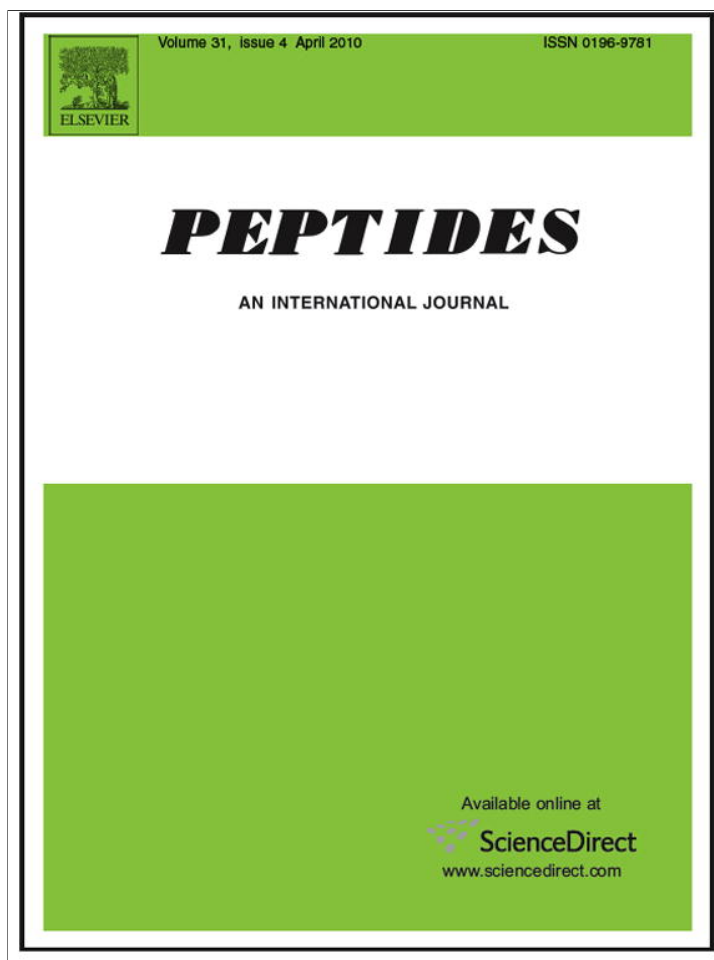


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## Antimicrobial peptides from the skin secretions of the South-East Asian frog *Hylarana erythraea* (Ranidae)

Nadia Al-Ghaferi<sup>a</sup>, Jolanta Kolodziejek<sup>b</sup>, Norbert Nowotny<sup>b</sup>, Laurent Coquet<sup>c,d</sup>, Thierry Jouenne<sup>c,d</sup>, Jérôme Leprince<sup>c,e</sup>, Hubert Vaudry<sup>c,e</sup>, Jay. D. King<sup>f</sup>, J. Michael Conlon<sup>a,\*</sup>

<sup>a</sup> Department of Biochemistry, Faculty of Medicine and Health Sciences, United Arab Emirates University, 17666 Al-Ain, United Arab Emirates

<sup>b</sup> Zoonoses and Emerging Infections Group, Clinical Virology, Department of Pathobiology, University of Veterinary Medicine, Vienna, Veterinärplatz 1, A-1210, Vienna, Austria

<sup>c</sup> European Institute for Peptide Research, University of Rouen, 76821 Mont-Saint-Aignan, France

<sup>d</sup> FRE3101 CNRS, University of Rouen, 76821 Mont-Saint-Aignan, France

<sup>e</sup> INSERM U-413, CNRS, University of Rouen, 76821 Mont-Saint-Aignan, France

<sup>f</sup> Rare Species Conservatory Foundation, St. Louis, MO 63110, USA

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## ABSTRACT

Peptidomic analysis of norepinephrine-stimulated skin secretions of the South-East Asian frog *Hylarana erythraea* (formerly *Rana erythraea* partim) has led to the identification of multiple peptides with antimicrobial activity. Structural characterization of the peptides demonstrated that they belong to the brevinin-1 (3), brevinin-2 (2), esculentin-2 (4), and temporin (1) families. The values in parentheses indicate the number of paralogs. In addition, a peptide (GVKSVLKGVAKTVALG MLNH<sub>2</sub>) was isolated that shows some structural similarity to the brevinin-2-related peptides (B2RP) previously isolated from North American frogs of the genus *Lithobates*. A synthetic replicate of the species B2RP showed broad-spectrum growth inhibitory activity against reference strains of *Escherichia coli* (MIC = 12.5 μM), *Staphylococcus aureus* (MIC = 12.5 μM) and *Candida albicans* (MIC = 50 μM) and was active against multidrug-resistant clinical isolates of *Acetivobacter baumannii* (MIC in the range 6–12.5 μM). The hemolytic activity of the peptide was relatively low (LC<sub>50</sub> = 280 μM). Phylogenetic analysis based upon the amino acid sequences of 47 brevinin-2 peptides from 17 Asian species belonging to the family Ranidae provides support for the placement of *H. erythraea* in the genus *Hylarana*.

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### 1. Introduction

Skin secretions from a variety of species of Anura (frogs and toads) contain peptides with broad-spectrum antibacterial and antifungal activities and the ability to permeabilize mammalian cells (reviewed in [9]). They constitute a component of the system of innate immunity that defends the animal against invasion by pathogenic microorganisms [19]. Such peptides have excited interest because of their potential for development into therapeutically valuable anti-infective agents [19,22] and for their value as phylogenetic markers for gaining insight into the evolutionary history of the different frog families [10,11]. The antimicrobial peptides themselves may be grouped together in families on the basis of limited similarities in amino acid sequence [6]. Skin secretions from a single species frequently contain several members of a particular peptide family with varying degrees of

potency and selectivity towards microorganisms that are presumed to have arisen from multiple duplications of an ancestral gene [38].

Skin secretions from frogs belonging to the family Ranidae (“true frogs”) have proved to be a particularly rich source of antimicrobial peptides and more than 200 such peptides from approximately 60 species have been described (reviewed in [10,11]). The Green Paddy frog *Hylarana erythraea* (Schlegel, 1837) (formerly classified as *Rana erythraea*) is a small (snout-vent length up to 45 mm for males and 75 mm for females), mainly nocturnal frog that is readily identified by a pair of cream-colored bands along the side of the body. It is widely distributed in South-East Asia (Cambodia, Laos, Vietnam, Java, Borneo, West and East Malaysia, Singapore, and Thailand) and has been introduced to the Philippines [17,25]. Populations in Northern India that were formerly assigned to *R. erythraea* are now included within *Hylarana tytleri* [26]. The animal’s natural habitats are tropical and subtropical moist lowland forests and freshwater marshes but the species has adapted well to human activity and is frequently found in agricultural areas, rural gardens, and irrigation ditches

\* Corresponding author. Tel.: +971 3 7137484; fax: +971 3 7672033.  
E-mail address: [jmconlon@uaeu.ac.ae](mailto:jmconlon@uaeu.ac.ae) (J.M. Conlon).

and ponds. The species is not currently considered to be threatened or endangered but declines due to loss of habitat and water pollution by agrochemicals have taken place [15].

This study describes the purification and structural characterization of 11 peptides with cytolytic activity against microorganisms from norepinephrine-stimulated skin secretions of *H. erythraea*. Nomenclature adopted for antimicrobial peptides from frogs of the Ranidae family follows recent guidelines [6]. Peptides from previously described families are given the suffix ER and isoforms are denoted by lower case letters e.g. brevinin-1ERa.

## 2. Materials and methods

### 2.1. Collection of skin secretions

All experiments with live animals were approved by the Animal Research Ethics Committee of UAE University and were carried out by authorized investigators. Five adult and sub-adult specimens of *H. erythraea* (weights 6–12 g; sexes unknown) were collected in central Vietnam and imported into the U.S. by a U.S. Fish and Wildlife Service licensed importer. Each animal was injected via the dorsal lymph sac with norepinephrine hydrochloride (40 nmol/g body weight) and placed in a solution (100 ml) of distilled water for 15 min. The frog was removed and the collection solution was acidified by addition of trifluoroacetic acid (TFA) (1 ml) and immediately frozen for shipment to U.A.E. University. The solutions containing the secretions were pooled and separately passed at a flow rate of 2 ml/min through 6 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA) connected in series. Bound material was eluted with acetonitrile/water/TFA (70.0:29.9:0.1, v/v/v) and freeze-dried. The material was redissolved in 0.1% (v/v) TFA/water (2 ml).

### 2.2. Peptide purification

The skin secretions, after partial purification on Sep-Pak cartridges, were injected onto a (2.2 cm × 25 cm) Vydac 218TP1022 (C-18) reversed-phase HPLC column (Grace, Deerfield, IL) 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 280 nm, and fractions were collected by hand. Purification of the peptides was monitored by incubating lyophilized aliquots of chromatographic effluent (100 μl) in Mueller–Hinton broth (50 μl) with an inoculum (50 μl of 10<sup>6</sup> colony forming units/ml) from a log-phase culture of reference strains of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) in 96-well microtiter cell-culture plates for 18 h at 37 °C in a humidified atmosphere of air. After incubation, the absorbance at 630 nm of each well was determined using a microtiter plate reader. Fractions associated with antimicrobial activity were successively chromatographed on a (1 cm × 25 cm) Vydac 214TP510 (C-4) column and a (0.46 cm × 25 cm) Vydac 218TP54 C-18 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 (semipreparative column) or 1.5 ml/min (analytical column).

### 2.3. 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). 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%. Amino acid composition analyses were performed by the University of Nebraska Medical Center Protein Structure Core Facility (Omaha, NE).

### 2.4. Peptide synthesis

*H. erythraea* B2-RP (GVIKSVLKGVAKTVALGMLNH<sub>2</sub>) was supplied in crude form by GL Biochem (Shanghai) Ltd. (China) and was purified to near homogeneity (> 98% purity) by reversed-phase HPLC on a (2.2 cm × 25 cm) Vydac 218TP1022 (C-18) column equilibrated with acetonitrile/water/TFA (28.0/71.9/0.1, v/v/v) at a flow rate of 6 ml/min. The concentration of acetonitrile was raised to 56% (v/v) over 60 min using a linear gradient. Absorbance was measured at 214 nm and 280 nm and the major peak in the chromatogram was collected manually. The identity of the synthetic peptide was confirmed by electrospray mass spectrometry.

### 2.5. Antimicrobial and hemolytic activities of B2RP

Minimum inhibitory concentrations (MIC) of synthetic *H. erythraea* B2RP against reference strains of *S. aureus* (ATCC 25923), *E. coli* (ATCC 25726), and *Candida albicans* (ATCC 90028), purchased from the American Type Culture Collection (Rockville, MD), were measured by standard microdilution methods [4,5]. In order to monitor the validity and reproducibility of the assays, incubations were carried out in parallel with increasing concentrations of ampicillin for reference strains of bacteria and amphotericin for *C. albicans* as previously described [13,14].

Five independent *Acinetobacter baumannii* strains isolated at four different hospitals in Abu Dhabi Emirate were included in the study. The strains were resistant to all antibiotics commonly used to treat *Acinetobacter* infections including cephalosporins, carbapenems, fluoroquinolones, aminoglycosides but remained sensitive to tigecycline and colistin. The clonal lineages of the strains have been described in a previous article [8] and are summarized in Table 1.

Hemolytic activity against human erythrocytes from a healthy donor was measured as previously described [8]. The LC<sub>50</sub> value was taken as the mean concentration of peptide producing 50% hemolysis in three independent experiments.

### 2.6. Cladistic analysis

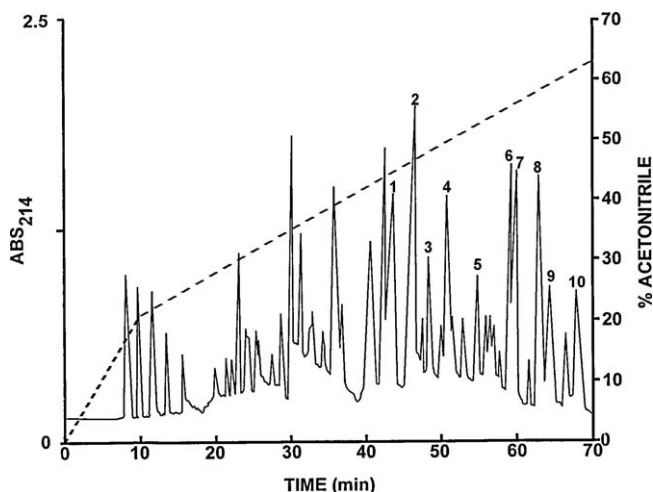
Cladistic analysis based upon the amino acid sequences of 47 brevinin-2 peptides from 17 Asian species belonging to the family Ranidae was performed using the neighbor-joining method [34]. The evolutionary distances were computed using the Poisson correction method [41] and are in the units of the number of amino acid substitutions per site. All positions containing alignment gaps were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 37 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 [36].

**Table 1**

Antimicrobial potencies of B2RP from *H. erythraea* against antibiotic-resistant nosocomial isolates of *Acinetobacter baumannii*.

| Strain | Origin  | Clonal lineage | MIC  |
|--------|---------|----------------|------|
| NM8    | Sputum  | Euro clone I   | 6    |
| NM35   | Trachea | Euro clone II  | 6    |
| NM75   | Wound   | Euro clone I   | 12.5 |
| NM109  | Trachea | Euro clone II  | 12.5 |
| NM124  | Urine   | Non-typable    | 12.5 |

Growth inhibitory activities of the peptides are expressed as minimum inhibitory concentrations (μM).



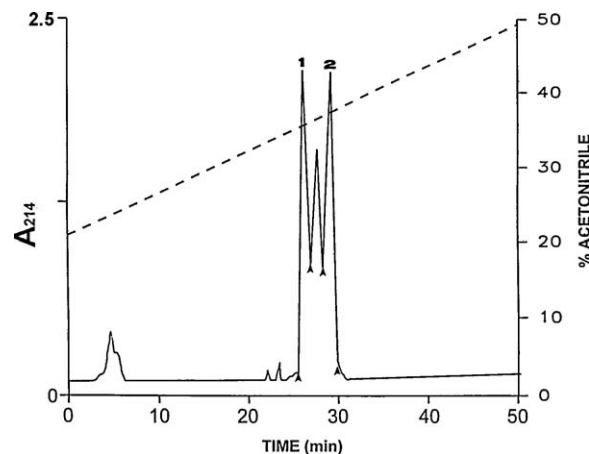
**Fig. 1.** Reversed-phase HPLC on a preparative Vydac C-18 column of skin secretions from *H. erythraea* after partial purification on Sep-Pak cartridges. The peaks designated 1–10 were associated with antimicrobial activity and were purified further. The dashed line shows the concentration of acetonitrile in the eluting solvent.

The stability of the tree was tested by bootstrap resampling analysis of 1000 replicates. The amino acid sequence of brevinin-2 from the European ranid frog *Pelophylax lessonae* (formerly *Rana*) (GIMDTLKNLAKTAGKALQSLNKASCKLSGQC) [33] was used as out-group to polarize the in-group taxa.

### 3. Results

#### 3.1. Purification of the peptides from *H. erythraea*

The elution profile on a preparative Vydac C-18 column of the norepinephrine-stimulated skin secretion from *H. erythraea* after partial purification on Sep-Pak cartridges, is shown in Fig. 1. Ten peaks (designated 1–10 in Fig. 1) were associated with differential antimicrobial activity. Under the condition of assay, material from peak 1 inhibited growth of *E. coli* only, peaks 8–10 inhibited growth of *S. aureus* only, and peaks 2–7 inhibited growth of both *E. coli* and *S. aureus*. Subsequent structural analysis showed that peak 1 contained brevinin-2ERa, peak 2: a mixture of esculentin-2ERa and brevinin-2ERb, peak 3: B2RP, peak 4: esculentin-2ERb, peak 5: esculentin-2ERc, peak 6: brevinin-1ERa, peak 7: esculentin-2ERc,



**Fig. 2.** Separation of esculentin-2ERa (peak 1) and brevinin-2ERb (peak 2) on a semipreparative Vydac C-4 column. The dashed line shows the concentration of acetonitrile in the eluting solvent and the arrowheads show where peak collection began and ended.

peak 8: brevinin-1ERb, peak 9: brevinin-1ERc, and peak 10: temporin-ERa.

The peptides were purified to near homogeneity by further chromatography on a semipreparative Vydac C-4 and an analytical Vydac C-18 column. The chromatographic methodology is illustrated by the separation of esculentin-2ERa and brevinin-2ERb on a Vydac C-4 column (Fig. 2).

#### 3.2. Structural characterization

The primary structures of the antimicrobial peptides isolated from *H. erythraea* were established by automated Edman degradation and their amino acid sequences are shown in Fig. 3. As there was some ambiguity with regard to identification of the C-terminal amino acid residue in the B2RP peptide, the sequence was confirmed by amino acid composition analysis [found: Thr 1.0 (1), Ser 1.1 (1), Gly 3.2 (3), Ala 2.1 (2), Val 3.7 (4), Met 1.0 (1), Ile 0.5 (1), Leu 3.1 (3), Lys 3.2 (3) residues/mol peptide]. Figures in parentheses show the number of residues predicted from the proposed sequences. The results of MALDI-TOF mass spectrometry were consistent with the proposed sequences and demonstrated the presence of a disulfide bridge in the brevinin-1, brevinin-2, and esculentin-2, peptides and the presence of a C-terminally  $\alpha$ -amidated residue at the C-termini of B2RP and temporin-ERa (Fig. 4).

|                 |  | [M+H] <sub>obs</sub> | [M+H] <sub>calc</sub> |
|-----------------|--|----------------------|-----------------------|
| Brevinin-2ERa   | GVVKDTLKSVAKTVALQLVNTAKCKLEKTC         | 3186.8               | 3186.8                |
| Esculentin-2ERa | GLFSLFKAGAKILGKTFLLKQAGKAGAEHLACKAANQC | 3789.6               | 3789.1                |
| Brevinin-2ERb   | GAIKETLKDFAKTVALGLVNTAKCKLEKTC         | 3192.1               | 3191.8                |
| B2RP-ERa        | GVIKSVLKGVAKTVALGML.NH <sub>2</sub>    | 1883.2               | 1883.2                |
| Esculentin-2ERb | GILNTLKNVGLGVLLKAGKAGALNAVLCKMNNNC     | 3295.8               | 3295.8                |
| Esculentin-2ERc | GILNTLKNVGLGVLLKSAGKAGALNAVLCKMNNSC    | 3299.4               | 3298.8                |
| Brevinin-1ERa   | FLPGLIKVAAGLIPKVCKFTNKC                | 2557.3               | 2557.5                |
| Esculentin-2ERd | SILTTLKDVGISVAKAAGSGVLKALLCKLNKNCEA    | 3527.0               | 3527.0                |
| Brevinin-1ERb   | FLPTLIKVAANVIPSIIICKFTGKC              | 2574.3               | 2574.5                |
| Brevinin-1ERc   | FLPTLIKVAADVIPSIIICKFTGKC              | 2575.6               | 2575.5                |
| Temporin-ERa    | FLPLIIGALSSLLPKIF.NH <sub>2</sub>      | 1841.1               | 1841.2                |

**Fig. 3.** Amino acid sequences, observed molecular masses ( $M_r$ , obs), and calculated molecular masses ( $M_r$ , calc) of the antimicrobial peptides isolated from skin secretions of *H. erythraea*.

Temporin

*H. erythraea* FLPLIIGALSSLLPKIF.NH<sub>2</sub>  
*H. guntheri* FFPLIFGALSSILPKIL.NH<sub>2</sub>  
*H. latouchii* FLPIALKALGSIPFKIL.NH<sub>2</sub>  
*R. sakuraii* a FLPVILPVIKLLNLGIL.NH<sub>2</sub>  
*R. sakuraii* b FLPVILPVIKLLSLGIL.NH<sub>2</sub>  
*R. tagoi* FLPVILPVIKLLSLGIL.NH<sub>2</sub>

B2RP

*H. erythraea* GVIKSVLKGVAK\*\*\*TVALGML.NH<sub>2</sub>  
*L. septentrionalis* GIWDTI\*KSMGKVFAGKILQNL.NH<sub>2</sub>  
*L. virgatipes* a GIWDTL\*KNVGKAVLGKVLENV.NH<sub>2</sub>  
*L. virgatipes* b SIWDTI\*KNVGKTVLGVLEIV.NH<sub>2</sub>

**Fig. 4.** A comparison of the primary structures of peptides belonging to the temporin and brevinin-2-related peptide (B2RP) families isolated from the skins of frogs from the genera *Hylarana*, *Lithobates* and *Rana*. The shaded residues are conserved between species. In order to maximize structural similarity, residue deletions denoted by (\*) have been introduced in some sequences.

3.3. Cladistic analysis

A phylogenetic tree based upon the amino acid sequences of the brevinin-2 peptides isolated from Asian frogs belonging to the family Ranidae (Fig. 5) was generated using the neighbor-joining method. The optimal tree with the sum of branch length = 8.26736162 is shown in Fig. 6. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

3.4. Cytolytic activity of *H. erythraea* B2RP

The minimum inhibitory concentration (MIC) of synthetic B2RP against *E. coli* was 12.5 μM, against *S. aureus* 12.5 μM, and against *C. albicans* 50 μM. The hemolytic activity against human erythrocytes (LC<sub>50</sub>) was 280 μM. The MIC values against the antibiotic-resistant clinical isolates of *A. baumannii* are shown in Table 1.

4. Discussion

This study has demonstrated that antimicrobial peptides present in norepinephrine-stimulated skin secretions of *H. erythraea* belong to the well-characterized brevinin-1, brevinin-2, esculentin-2, and temporin families (Fig. 3). Brevinin-1 and brevinin-2 peptides were first identified in the skin of *R. brevipoda* porsa (reclassified as *Pelophylax porosus*) [24] but subsequent work has shown that members of the brevinin-1 family are widely distributed in both Eurasian and N. American species whereas, to-date, brevinin-2 peptides have only been found in Eurasian ranids [6,11]. Similarly, esculentin-2 peptides were first identified in the Eurasian frog *R. esculenta* (now reclassified as a hybrid between *P. lessonae* and *P. ridibundus*) [33] but have subsequently been detected in the skins of several species that belong to the genera *Lithobates*, *Odorrana*, *Pelophylax* and *Rana* [6]. There was insufficient pure material to determine the MIC values of the *H. erythraea* peptides isolated in this study but peptides of the brevinin-1, brevinin-2, and esculentin-2 are generally active against Gram-positive and Gram-negative bacteria but their therapeutic potential is often limited by high hemolytic activity against human erythrocytes [10].

The 17 amino acid residue, very hydrophobic peptide FLPLIIGALSSLLPKI F.NH<sub>2</sub> has been designated a member of the temporin family and termed temporin-ERa. Members of the temporin family are widely distributed in both New World and Eurasian species belonging to the family Ranidae [6,22] and, although characterized

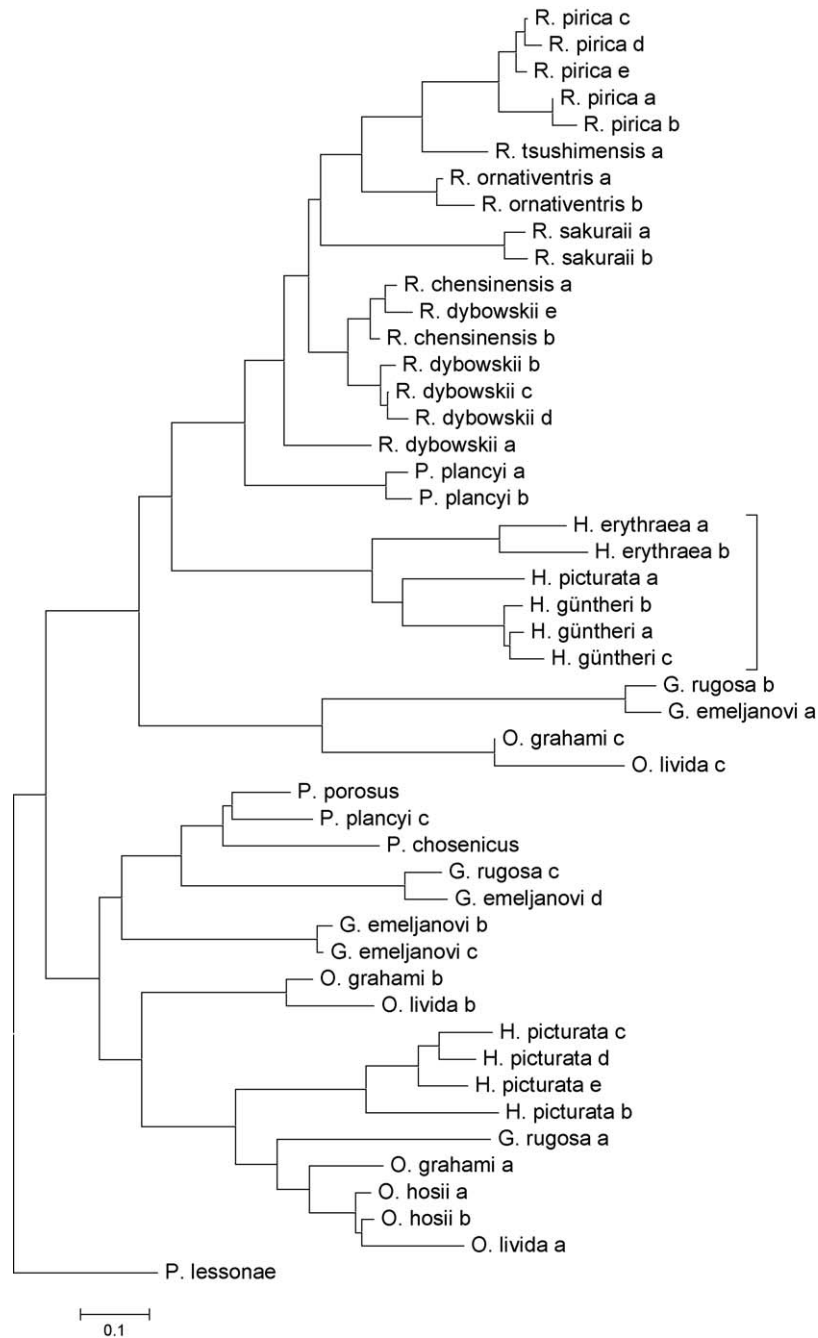
Brevinin-2

*H. erythraea* a GVVKDT\*\*\*LKSVAKTVA\*\*\*\*LQLVNTAKCKLEKTC  
*H. erythraea* b GAIKET\*\*\*LKDFAKTVA\*\*\*\*LGLVNTAKCKLEKTC  
*H. picturata* a GAIKDA\*\*\*LKGAAKTVA\*\*\*\*VELLKAQCKLEKTC  
*H. picturata* b GFKGAFKPNVMPGIKASAG\*\*\*\*KSALNALACKIDKSC  
*H. picturata* c GLDSSFKNAMIGIATKSAG\*\*\*\*KTALNKIACKIDKTC  
*H. picturata* d GFLDSSFKNAMIGVAKSAG\*\*\*\*KTALNTLACKIDKTC  
*H. picturata* e GFLDSSFKNAMIGVAKSVG\*\*\*\*KTALSTLACKIDKSC  
*H. guntheri* a GVITDA\*\*\*LKGAAKTVA\*\*\*\*AELLRKAHCKLTNSC  
*H. guntheri* b GVIIDT\*\*\*LKGAAKTVA\*\*\*\*AELLRKAHCKLTNSC  
*H. guntheri* c GVIIDT\*\*\*LKGVAKTVA\*\*\*\*AELLRKAHCKLTNSC  
*P. porosus* GLDLSLKGFAATAGKGVL\*\*\*\*QSLLSTASCCLAKTC  
*P. chosonicus* GLDLSIKGMAISAGKGA\*\*\*\*QNLLKVASCKLDKTC  
*P. plancyi* a GILTDI\*\*\*LKGAAKNVA\*\*\*\*GVLLDLKCKITGGC  
*P. plancyi* b GILLNT\*\*\*LKGAAKNVA\*\*\*\*GVLLDLKCKITGGC  
*P. plancyi* c GLMDSLKGLAATAGKTVL\*\*\*\*QGLLKTASCCKLEKTC  
*R. chensinensis* a GLFSVVGKVLKAVGKNVAKNVGSSLEKCKISGGC  
*R. chensinensis* b GLFSVVGKVLKAVGKNVA\*\*\*\*GSLEKCKISGGC  
*R. dybowskii* a GLLSAVKGVKLGAGKNVA\*\*\*\*GSLMDLCKCLFGGC  
*R. dybowskii* b GLFDVVKGVKLGAGKNVA\*\*\*\*GSLEQLKCKLSGGC  
*R. dybowskii* c GLFDVVKGVKLGAGKNVA\*\*\*\*GSLEQLKCKLSGGC  
*R. dybowskii* d GIPDVKGVKLGAGKNVA\*\*\*\*GSLEQLKCKLSGGC  
*R. dybowskii* e GLFSVVTGVLKAVGKNVAKNVGSSLEQLKCKISGGC  
*R. pirica* a GLMSLFGKVLKTAGKHI FKNVGGSLDQAKCKITGEC  
*R. pirica* b GLMSLFRGVLKTAGKHI FKNVGGSLDQAKCKITGEC  
*R. pirica* c GLMSVLKGVKLTAGKHI FKNVGGSLDQAKCKISGQC  
*R. pirica* d GLMSVLKGVKLTAGKHI FKNVGGSLDQAKCKITGQC  
*R. pirica* e GLLSVLKGVKLTAGKHI FKNVGGSLDQAKCKISGQC  
*R. sakuraii* a GLFSAFKVKGNVKNVA\*\*\*\*GSLMDNLCKVSGEC  
*R. sakuraii* b GLFNVFKKVGKNVKNVA\*\*\*\*GSLMDNLCKVSGEC  
*R. ornativentris* a GLFNVFKGALKTAGKHVA\*\*\*\*GSLNLQKCKVSGGC  
*R. ornativentris* b GIFNVFKGALKTAGKHVA\*\*\*\*GSLNLQKCKVSGGC  
*R. tsushimensis* a GIMSLFKGVKLTAGKHVA\*\*\*\*GSLVDQKCKITGGC  
*G. rugosa* a GLLNTPKDWAI SIKAGG\*\*\*\*KGVLTTLSCCKLDKSC  
*G. rugosa* b SLFSLIKAGAKPLGNLL\*\*\*\*KQGAQYAAACKVSKTC  
*G. rugosa* c GILDSPKQFAKGVGKDLIKGAAQGVLSLTVSCCKLAKTC  
*G. emeljanovi* a SLFSLIKAGAKPLGNLL\*\*\*\*KQGACYAACKASKQC  
*G. emeljanovi* b GIMSIVKDVAKNAAKEAA\*\*\*\*KGLSTLSCCKLAKTC  
*G. emeljanovi* c GIMSIVKDVAKTAKEAA\*\*\*\*KGLSTLSCCKLAKTC  
*G. emeljanovi* d GILDTLKQFAKGVGKDLVKGAAQGVLSLTVSCCKLAKTC  
*O. hosii* a GLDLSLKNLAINAAKAG\*\*\*\*QSVLNTLSCCKLSKTC  
*O. hosii* b GLLDTLKNMAINAAGG\*\*\*\*QSVLNTLSCCKLSKTC  
*O. grahami* a GLLDTPKLNALNAAKAG\*\*\*\*QSVLNTLSCCKLSKTC  
*O. grahami* b GVLGTVKNLLIGAGKSA\*\*\*\*QSVLNTLSCCKLSNDC  
*O. grahami* c GLFTLIKGAALKIGKTVAKEAGKTLGELMACKITNQC  
*O. livida* a SFLDTLKNLAI SAAGKAG\*\*\*\*QSVLSTLSCCKLSKTC  
*O. livida* b SVLGTVKNLLIGAGKSA\*\*\*\*QSVLNTLSCCKLSNSC  
*O. livida* c GVFTLIKAGATQIGKTLGKELGKTLGELMACKITNQC

**Fig. 5.** Primary structures of the brevinin-2 peptides isolated from the skins of Asian frogs from the genera *Glandirana*, *Hylarana*, *Odorrana*, *Pelophylax*, and *Rana* were used to create the phylogenetic tree shown in Fig. 6. In order to maximize structural similarity, residue deletions denoted by (\*) have been introduced in some sequences.

by extreme variability in amino acid sequence, generally contain 13 amino acid residues. As shown in Fig. 4, temporin peptides with >13 residues have been identified in the skins of *H. guntheri* [14,40], *H. latouchii* [39] and *R. sakuraii* [35] and the intestine of *R. tagoi* [20]. Although there was again insufficient pure material to determine the MIC, temporin-ERa in common with most members of the family that contain a single basic residue [31], showed greater growth inhibitory activity against the Gram-positive bacteria *S. aureus* than against the Gram-negative bacteria *E. coli*.

The taxonomy of frogs belonging to the extensive family Ranidae has undergone a series of major revisions in recent years but many issues remain to be resolved. Analyses based upon molecular and morphological criteria demonstrate that the former extensive genus *Rana*, comprising in excess of 250 species, did not constitute a monophyletic group [3,18] with the result that many well known species have been reclassified. Current recommendations by Frost [17] divide the 338 species in the family Ranidae into 16 genera with the genus *Rana* being retained for a more restricted group of 48 species from Eurasia and North America. It must be pointed out, however, that other established taxonomists have claimed that many species reclassifications are both arbitrary and premature and it has been suggested that the genus *Rana* should be retained for all North American members of the family [27]. Although the phylogeny of the Japanese [12,37] and Chinese [2]



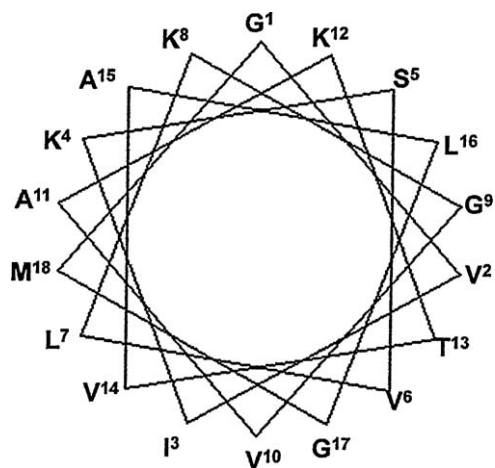
**Fig. 6.** A phylogenetic tree based upon the amino acid sequences of the brevinin-2 peptides isolated from the skins of Asian frogs belonging to the family Ranidae and shown in Fig. 5. Brevinin-2 from the European frog *P. lessonae* was used as out-group.

brown frogs has been studied in some detail, evolutionary relationships among the diverse populations of other ranid frogs in China, India, and South-East Asia are incompletely understood.

The former *Rana erythraea* has now been reclassified along with 85 other species from tropical Asia and the African savannas in the extensive genus *Hylarana* [17]. As shown in Fig. 6, cladistic analysis based upon the amino acid sequence of 47 brevinin-2 peptides from 17 Asian species has provided support for this placement. In the phylogenetic tree generated by the neighbor-joining method, *H. erythraea* is the sister-group to a well-defined clade, designated by the bar in Fig. 6, containing the Malaysian frog *H. picturata* [13] and the Chinese frog *H. güntheri* [14]. Consistent with phylogenetic analyses based upon the nucleotide sequences of mitochondrial genes, the Japanese brown frogs *R. pirica*, *R. tsushimensis*, *R. ornativentris*, and *R. sakuraii* form a well-defined separate clade

[37] and the Chinese brown frog *R. chensinensis* is sister-group to *R. dybowskii* [2]. Evolutionary relationships between species belonging to the genera *Glandirana*, *Odorrana*, and *Pelophylax* are less well defined in this cladogram and it is clearly necessary to obtain structural data on brevinin-2 peptides from more species.

The assignment of a newly discovered antimicrobial peptide to a particular previously described family is a somewhat arbitrary process and the evolutionary relationship of the peptide GVIKSVLKGVAKTVALGMLNH<sub>2</sub> to other frog skin peptides is not entirely clear. To-date, brevinin-2 peptides have not been detected in any North American ranid species [11] but C-terminally  $\alpha$ -amidated peptides with some structural similarity to brevinin-2, but lacking the C-terminal cyclic heptapeptide domain (Cys-Lys-Xaa<sub>4</sub>-Cys) were isolated from skin secretions from the New World species, the mink frog *Rana septentrionalis* (reclassified as



**Fig. 7.** A Schiffer–Edmundson helical wheel representation of the brevinin-2-related peptide isolated from *H. erythraea* illustrating the amphipathic nature of the  $\alpha$ -helix.

*Lithobates septentrionalis* [1] and the carpenter frog *Rana virgatipes* (reclassified as *Lithobates virgatipes*) [7]. As shown in Fig. 4, the peptide from *H. erythraea* shows limited structural similarity to these peptides and so has been designated a brevinin-2-related peptide (B2RP). The vast majority of frog skin antimicrobial peptides investigated to-date adopt an amphipathic  $\alpha$ -helical conformation in a membrane-mimetic solvent such as 50% trifluoroethanol–water [29]. Secondary structure predictions by the method of Rost et al. [30] indicate that the B2RP has a high probability of forming a stable  $\alpha$ -helical conformation between residues (2–16) and a Schiffer–Edmundson wheel representation of the molecule [32] demonstrates the amphipathic nature of the helix (Fig. 7). The hydrophilic face of the helix in B2RP is associated with a net charge of +4 (Lys<sup>4</sup>, Lys<sup>8</sup>, and Lys<sup>12</sup> and the  $\alpha$ -amino group of Gly<sup>1</sup>). *H. erythraea* B2RP, in common with the putative ortholog from *L. septentrionalis* [1], showed relatively high growth inhibitory potency against Gram-positive and Gram-negative bacteria and against the opportunistic yeast pathogen *C. albicans*.

Multidrug-resistant strains of *A. baumannii* have emerged in all parts of the world and constitute a major threat to public health [16,28]. The Gram-negative opportunistic pathogen is responsible for a range of infections including pneumonia, bacteremia, meningitis, peritonitis and infections of the urinary tract and skin that are typically encountered in immunocompromised and critically ill patients in intensive-care units [16]. The bacterium is remarkable for its ability to acquire new determinants of resistance severely limiting treatment options [21,23] so that there is clearly a need for new types of antimicrobial agent to which the pathogen has not been exposed for treatment of multidrug-resistant *A. baumannii* infections. A previous study [8] demonstrated that B2RP from *L. septentrionalis* potently (MIC = 3–6  $\mu$ M) inhibited the growth of nosocomial isolates of multidrug-resistant *A. baumannii* but its therapeutic potential was limited by moderately high hemolytic activity against human erythrocytes (LC<sub>50</sub> = 90  $\mu$ M). As shown in Table 1, B2-RP from *H. erythraea* displayed a 2-fold lower potency against the same clinical isolates but this disadvantage was offset by appreciably lower hemolytic activity (LC<sub>50</sub> = 280  $\mu$ M). Consequently, this peptide shows potential for development in a therapeutically valuable agent for the systemic treatment of *A. baumannii* infections.

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