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ORIGINAL ARTICLE

Antimicrobial peptide diversity in the skin of the torrent frog, *Amolops jingdongensis*

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Abstract Antimicrobial peptide diversity has been found in some amphibians. The diversity of antimicrobial peptides may have resulted from the diversity of microorganisms encountered by amphibians. Peptidomics and genomics analyses were used to study antimicrobial peptide diversity in the skin secretions of the torrent frog, *Amolops jingdongensis*. Thirty-one antimicrobial peptides belonging to nine groups were identified in the skin secretions of this frog. Among them, there are two novel antimicrobial groups (jingdongin-1 and -2) with unique structural motifs. The other seven groups belong to known antimicrobial peptide families, namely brevinin-1, brevinin-2, odorranain-F, esculentin-2, temporin, amolopin-3, and ranacyclin. Combined with previous reports, more than 13 antimicrobial peptide groups have been identified from the genus *Amolops*. Most of these antimicrobial peptide groups are also found in amphibians belonging to the genus *Rana* or *Odorrana* which suggests a possible evolutionary connection among *Amolops*, *Rana*, and *Odorrana*. Two novel antimicrobial groups (jingdongin-1 and -2) were synthesized and their antimicrobial activities were assayed. Some of them showed strong antimicrobial abilities against microorganisms including Gram-negative and -positive bacteria, and fungi. The extreme diversity of antimicrobial peptides in the *Amolops* amphibians was demonstrated. In addition, several novel peptide templates were provided for antimicrobial agent design.

Keywords Antimicrobial peptides · Diversity · Amphibian · Skin · *Amolops*

Introduction

Amphibian skins are multifunctional organs that participate in defense, respiration, and water regulation. They play key roles in the everyday survival of amphibians and their ability to exploit a wide range of habitats and ecological conditions. Amphibian skins are susceptible to biotic or abiotic injuries, such as predation, parasitization, microorganism infection, and physical harm including aseptic wounds and radiation (Clarke 1997). Therefore, they are endowed with an excellent chemical defense system composed of a variety of skin defensive chemicals (Clarke 1997). Gene-encoded defensive peptides are main components of the chemical array in amphibian skins. These defensive peptides include antimicrobial peptides, analgesic peptides (dermorphin, dermenkephalin, and deltorphins), algesic peptides (bradykinin, tachykinin, and cholecystokinin), protease inhibitors, lectins, antioxidant peptides, and neurotoxins. Antimicrobial peptides act as anti-infectives and have been found in many amphibians (Zasloff 1987; Conlon et al. 2004; Duda et al. 2002; Lai et al. 2002; Li et al. 2007). Analgesic peptides may attenuate suffering. Several analgesic peptides with a D-amino acid in position 2 of their sequence have been

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found in the skin secretions of the South American frogs *Phyllomedusa*; these peptides act on the mu- and delta-opioid receptors (Amiche et al. 1998). A large number of algesic peptides including bradykinin, tachykinin, and cholecystokinin have been identified in amphibian skins (Erspamer et al. 1981; Severini et al. 2002; Li et al. 2006; Yasuhara et al. 1979; Liu et al. 2007; Conlon 1999). These peptides may take part in alarm behavior during attack and help the host to escape from injury. Protease inhibitors possibly inhibit parasite infection. More than 20 protease inhibitor peptides have been identified in amphibians (Yan et al. 2011). Antioxidant peptides exert free radical scavenging and radioprotective ability in amphibian skins. The first identification of amphibian antioxidant peptides was reported by Yang et al. (2009). A large number of antioxidant peptides belonging to 11 different groups with variable structures were isolated from the skin secretions of *Rana pleuraden* (Yang et al. 2009). Frog skins maintain redox homeostasis by using antioxidant peptides with rapid radical scavenging ability (Liu et al. 2010). Neurotoxins may take part in the defense against predators. Gene-encoded neurotoxin was first isolated from the tree frog *Hyla annectans* (You et al. 2009). Recently, several other gene-encoded neurotoxins were isolated from the tree frog *Hyla simplex* (Wu et al. 2011). These neurotoxins inhibit tetrodotoxin-sensitive voltage-gated sodium channels (You et al. 2009; Wu et al. 2011). The only known lectin from amphibian skin, odorranalectin, was isolated from the frog skin secretions of *Odorrana grahami*. This lectin could synergize with amphibian antimicrobial peptides and take part in anti-microbial defense as antimicrobial peptides do (Li et al. 2008).

Antimicrobial peptides expressed in amphibian skins act as the first line in defense against microorganism infection. They have important roles in innate immunity (Boman 1991; Zasloff 1992). They are fast-acting agents to kill microorganisms. Our previous works have indicated that antimicrobial peptides rapidly exert their antimicrobial activities: they just took less than 1 h to kill microorganisms at a concentration of 2, 4, or 10 times the minimal inhibitory concentration (MIC), whereas antibiotics took at least 6 h (Wang et al. 2008a; Yan et al. 2012). Another feature of antimicrobial peptides is that they have little potential to induce microorganism resistance. On the basis of these features, antimicrobial peptides have attracted intensive attention as novel clinical anti-infective candidates. The diversity of antimicrobial peptides provides much opportunity to identify templates for the development of anti-infective agents. *Amolops* frogs have special living environments and have been proven to contain novel antimicrobial peptide groups with unique structure in our previous works (Lu et al. 2006; Wang et al. 2008b). Here, the antimicrobial peptide diversity of *A. jingdongensis* skin was investigated.

Materials and methods

Collection of frog skin secretions

Adult specimens of *A. jingdongensis* of both sexes ($n = 20$; weight range 25–35 g) were collected in Yunnan Province of China. Skin secretions were collected as per our previous method (Wang et al. 2010). Frogs were stimulated with volatilized anhydrous ether immersed in absorbent cotton, and their skin surface was seen to exude secretions. Skin secretions were washed with 0.1 M NaCl solution (containing protease inhibitor cocktail, Sigma). The collected solutions containing skin secretions (1,000 ml total volume) were quickly centrifuged and the supernatants were lyophilized. All the experiments were approved by Kunming Institute of Zoology, Chinese Academy of Sciences.

Peptide purification

A 1.5-g lyophilized skin secretion sample of *A. jingdongensis* had a total absorbance at 280 nm of 500. A 0.5-g aliquot of lyophilized crude sample was dissolved in 10 ml 0.1 M phosphate buffer, pH 6.0 (PBS) and centrifuged at 5,000*g* for 10 min. The supernatant was applied to a Sephadex G-50 (Superfine, Amersham Biosciences; diameter 2.6 cm; length 100 cm) gel filtration column equilibrated with 0.1 M PBS. Elution was performed with the same buffer and fractions of 3.0 ml were collected. The absorbance of the elute was monitored at 280 nm. The antimicrobial activity was determined as described below. The fractions with antimicrobial activity were pooled and applied onto a C_{18} reversed-phase high-performance liquid chromatography column (RP-HPLC, Hypersil BDS C_{18}, 25 × 0.46 cm) for primary separation using the acetontrile gradient indicated in Fig. 1b. The peptide fractions exhibiting antimicrobial activity were pooled, lyophilized, and re-suspended in 2 ml 0.1 M PBS, and purified further by C_{18} RP-HPLC (Hypersil BDS C_{18}, 25 × 0.46 cm) using the conditions indicated in Fig. 1c–f.

Structural analysis

The purified antimicrobial peptides obtained by RP-HPLC were analyzed by a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF–MS) AXIMA CFR (Kratos Analytical) in positive ion and linear mode according to the recommended operation parameters (ion acceleration voltage 20 kV, accumulation

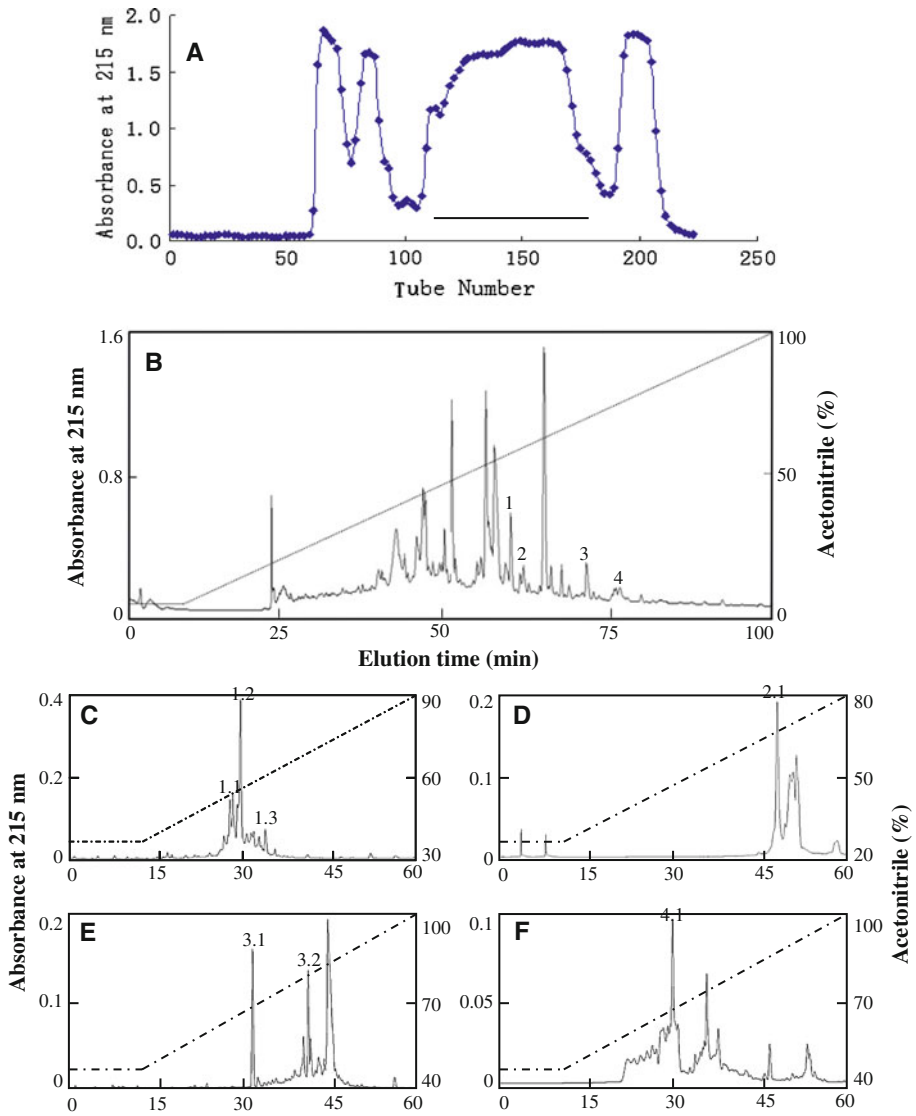


Fig. 1 Purification of antimicrobial peptides from skin secretions of *A. jingdongensis*. **a** Lyophilized skin secretion sample of *A. jingdongensis* was subjected to Sephadex G-50 gel filtration; the elution was performed with 0.1 M PBS at a flow rate of 0.3 ml/min. **b** Fraction III (indicated by the *horizontal bar* in **a**) was applied onto an RP-HPLC column (Hypersil BDS C_{18}, 25 × 0.46 cm). Elution was performed at a flow rate of 0.7 ml/min with the indicated gradients of acetonitrile in 0.1 % (v/v) trifluoroacetic acid (TFA) in water. **c–f** Eluted fractions corresponding to peaks *1–4* in Fig. 1b were separately subjected to further purification by C_{18} RP-HPLC. Peaks *1.1*, *1.2*, *1.3*, *2.1*, *3.1*, *3.2*, and *4.1* correspond to the purified antimicrobial peptides. The elution was performed at a flow rate of 0.7 ml/min with the indicated gradients of acetonitrile in 0.1 % (v/v) TFA in water

time of a single scan 50 s). The polypeptide mass standard (Kratos Analytical) was used as external standard. The accuracy of mass determinations was within 0.1 %. Amino acid sequences of the purified peptides were determined by automated Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491.

cDNA library construction

The dorsal skin was collected from one adult male frog of *A. jingdongensis* (30 g). Total RNA was extracted from the skin of the frog with TRIzol reagent (Life Technologies, Ltd.). A SMART^{TM} PCR cDNA synthesis kit purchased from Clontech (Palo Alto, CA) was used to synthesize the cDNA. Two primers, namely 3′ SMART CDS Primer II A, 5′-AAGCAGTGGTATCAACGCAGAGTACT (30) N-1 N-3′ (N = A, C, G, or T; N-1 = A, G, or C), and SMART II A oligonucleotide, 5′-AAGCAGTGGTATCAACGCAGAGT ACGCGGG-3′, were used for the first strand synthesis. The second strand was amplified using Advantage polymerase by 5′ PCR primer II A, 5′-AAGCAGTGGTATCAACGCAG AGT-3′. A directional cDNA library was constructed with a plasmid cloning kit (SuperScript^{TM} Plasmid System, GIBCO/BRL) according to manufacturer's instructions, producing a library of about 1.5 × 10^6 independent colonies.

cDNA screening

A PCR-based method for high stringency screening of DNA libraries was used for oligonucleotide primers, S_1 (5′-CCAAA(G/C)ATGTTCACC(T/A)TGAAGAAA(T/C)-3′, in the sense direction, a specific primer designed according to the signal peptide sequences of antimicrobial peptides from ranid frogs (Li et al. 2007), and primer II A as

mentioned in "SMART cDNA synthesis" in the antisense direction were used in PCR reactions. All the oligonucleotide primers for PCR were prepared with a DNA synthesizer (model 381A, Applied Biosystems). The PCR conditions were 2 min at 94 °C, followed by 30 cycles of 10 s at 92 °C, 30 s at 52 °C, 60 s at 72 °C. DNA sequencing was performed on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

Antimicrobial assays

Several bacterial and fungal strains used in antimicrobial assays, Gram-positive bacterium *Staphylococcus aureus* (ATCC 2592), *Bacillus subtilis* (ATCC 6633), Gram-negative bacteria *Escherichia coli* (ATCC 25922), *B. dysenteriae*, and fungus *Candida albicans* (ATCC 2002), were obtained from Kunming Medical College. Bacteria were first grown in LB (Luria–Bertani) broth to an absorbance of 0.8 at 600 nm. A 10-µl aliquot of the bacteria culture was then taken and added to 8 ml of fresh LB broth with 0.7 % agar and poured over a 90-mm Petri dish containing 25 ml of 1.5 % agar in LB broth. After the top agar hardened, a 20-µl aliquot of the test sample filtered through a 0.22-µm Millipore filter was dropped onto the surface of the top agar and completely dried before being incubated overnight at 37 °C as per our previous method (Lai et al. 2002). If an examined sample contained antimicrobial activity, a clear zone was observed on the surface of the top agar representing inhibition of bacterial growth. MIC was determined in liquid LB medium by incubating the bacteria in LB broth with the tested sample at different concentrations. The MIC at which no visible growth occurred was recorded.

Hemolytic testing

Hemolytic tests were performed using rabbit red cells in Alsever's solution (in g/l: NaCl, 4.2; citric acid·3Na·2H_2O, 8.0; citric acid·H_2O, 0.55; D-glucose, 20.5) according to the method reported by Bignami (1993). The antimicrobial peptide samples were serially diluted with Alsever's solution and incubated with red cells at 37 °C for 30 min. Red cells were centrifuged and the absorbance of the supernatant was measured at 595 nm. 100 % hemolysis was determined by adding 1 % Triton X-100 to a sample of cells.

Synthetic peptides

All of the peptides used for antimicrobial and hemolytic assays were synthesized by GL Biochem (Shanghai) Ltd. (Shanghai, China) and analyzed by HPLC and mass spectrometry to confirmed purity higher than 98 %.

Results

Purification of antimicrobial peptides

The supernatant of *A. jingdongensis* skin secretions was divided into four fractions by the Sephadex G-50 gel filtration as illustrated in Fig. 1a. The antimicrobial activity was concentrated in the fraction III as indicated by a horizontal bar. Fraction III was purified by C_{18} RP-HPLC as illustrated in Fig. 1b and more than 50 peaks were eluted. Fractions corresponding to peaks 1–4 (Fig. 1b) exhibited antimicrobial activity and they were further purified by C_{18} RP-HPLC as illustrated in Fig. 1c–f, respectively. Seven purified peptides (marked 1.1–1.3, 2.1, 3.1, 3.2, and 4.1 in Fig. 1c–f, respectively) were found to have antimicrobial activity.

Structural characterization

The seven purified peptides were subjected to mass spectrometry analysis and Edman degradation sequencing. The amino acid sequences were determined to be peptide 1.1, FLP LAVSLAANFLPKLFCKITKKC; peptide 1.2, FLPIVTG LLSSLL; peptide 1.3, FLPIVENCSLVCWENNQKC; peptide 2.1, GFMDTAKNVAKNVAVTLIDKLRCKVTGGC; peptide 3.1, FLSTLLKVAFKVVPTLFCPITKKC; peptide 3.2, FLPLFLPKIICVITKKC; and peptide 4.1, GIFSIIK-TAAKFLGKNLLKEAGKAGMEHLACKAKNEC. Among these peptides, only peptide 1.2 (named temporin-AJ1) has no half-cysteine in its amino sequence. BLAST search indicated that peptide 1.2 belongs to the antimicrobial peptide group of temporin containing an amidated residue at the C-terminus. Mass spectrometry analysis gave an observed mass of 1,071.5, which matched well with the calculated mass of peptide 1.2 with an amidated leucine (Fig. 2). The other six peptides have 2–3 half-cysteines, which possibly form one disulfide bridge. The results from mass spectrometry analysis and BLAST search suggest that there is an intramolecular disulfide bridge in these peptides (Fig. 2). Peptide 1.1 (named brevinin-1-AJ1) and 3.1 (named brevinin-1-AJ2) are two novel members of the antimicrobial group of brevinin-1. Peptide 2.1 (named odorranain-F-AJ2) belongs to the antimicrobial group of odorranain-F. Peptide 4.1 (named esculentin-2-AJ1) belongs to the antimicrobial group of esculentin-2. Peptide 1.3 (named jingdongin-1) and 3.2, which contain unique primary structures, showed no similarity with known peptides (named jingdongin-2). They are two novel groups of peptides. Peptide 1.3 contains 17 residues with a theoretical isoelectric point of 9.39. Peptide 3.2 contains 19 residues with a theoretical isoelectric point of 4.53. There are two acidic and one basic residue in peptide 3.2. The disulfide segments (so-called *Rana* box) comprising seven residues found in many

Fig. 2 Antimicrobial diversity in *A. jingdongensis* skin. *Asterisks* indicate identical amino acid residues. *CM* calculated mass of purified antimicrobial peptide from the skin secretions of *A. jingdongensis*, *OM* observed mass by mass spectrometry analysis

amphibian antimicrobial peptide groups also existed in both peptide 1.3 and 3.2.

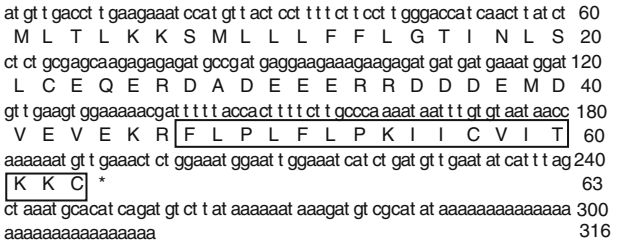
cDNA cloning

Thirty-one cDNA sequences (GenBank accession numbers JQ681272–JQ681301) encoding different antimicrobial peptides, including the seven purified peptides mentioned above, were cloned. The 31 peptides could be assembled into nine divergent groups (Fig. 2). Two groups (jingdongin-1 and -2) are novel peptide families. The precursors encoding jingdongin-1 and -2 are illustrated in Fig. 3. Their precursors contain 63 and 65 amino acid residues, respectively. As other antimicrobial peptide precursors, the precursors of jingdongin-1 and -2 are composed of signal peptide, acidic spacer peptide, mature peptide located at the C-terminus, and a predicted enzymatic processing site positioned between the spacer peptide and mature peptide. The other seven groups belong to known antimicrobial peptide families, namely brevinin-1, brevinin-2, odorranain-F, esculentin-2, temporin, amolopin-3, and ranacyclin. Among them, the amino acid sequence of mature temporin-AJ3, -4, -6, and -10 is identical to that of temporin-AL-I, -j, -b, and -a found in *A. loloensis*, respectively (Wang et al. 2010). The amino acid sequence of mature odorranain-F-AJ2 is identical to that of jindongenin-1a (Chen et al. 2012). In fact, jindongenin-1a belongs to the antimicrobial family odorranain-F found in *O. grahami* (Li et al. 2007) although it was named as a new group in a recent publication (Chen et al. 2012). Most of the antimicrobial peptides reported here are cationic peptides with net positive charge but brevinin-1-AJ3 and jingdongin-2 are anionic peptides with net negative charge.

Antimicrobial assays

Two novel antimicrobial groups (jingdongin-1 and -2), brevinin-1-AJ1, and temporin-AJ8 were synthesized and their antimicrobial activities were assayed as listed in Table 1. Three of them exerted antimicrobial activity. Brevinin-1-AJ1 and temporin-AJ8 showed antimicrobial activity against all of the tested microorganisms. *E. coli* is not sensitive to jingdongin-2. Brevinin-1-AJ1 had the strongest antimicrobial activity among the groups tested.

Jingdongin-1



Jingdongin-2

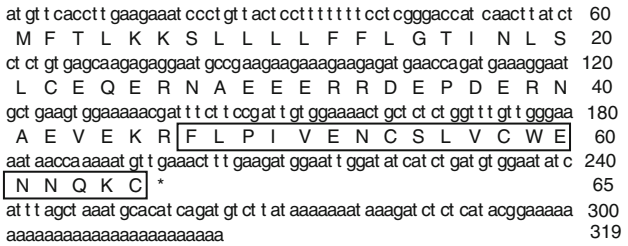


Fig. 3 cDNA sequences encoding precursors of jingdongin-1 (**a**) and -2 (**b**). The *asterisk* indicates a stop codon. Mature peptide is *boxed*

The MICs of brevinin-1-AJ1 against the five microorganism strains were 18.75, 9.38, 1.17, 4.7, and 4.7 μg/ml. Jingdongin-2 showed no antimicrobial activity against any of the tested microorganisms, which might be the result of its net negative charge.

Hemolytic activity

Rabbit red blood cells were used to evaluate the hemolytic capability of four antimicrobial peptides from the torrent frog (Table 2). Brevinin-1-AJ1, which had the strongest antimicrobial activity, showed the strongest hemolytic activity. At a concentration of 100 and 200 μg/ml, the hemolysis induced by brevinin-1-AJ1 was 83.05 and 87.65 %, respectively. Jingdongin-2 showed little hemolytic activity on rabbit blood red cells. At a concentration of 200 μg/ml, it only induced 3.7 % hemolysis. Temporin-AJ8 and jingdongin-1 also induced low hemolytic rates.

Table 1 Antimicrobial activity of antimicrobial peptides from *A. jingdongensis*

Microorganism	MIC (μg/ml)			
	Brevinin-1-AJ1	Temporin-AJ8	Jingdongin-1	Jingdongin-2
Gram-negative bacteria				
E. coli ATCC 25922	18.75	70	NA	NA
B. dysenteriae	9.38	18.75	35	NA
Gram-positive bacteria				
S. aureus ATCC 2592	1.17	4.7	4.7	NA
B. subtilis ATCC 6633	4.7	9.38	9.38	NA
Fungi				
C. albicans ATCC 2002	4.7	37.5	18.75	NA

Data represent mean values of three independent experiments performed in duplicate

MIC minimal inhibitory concentration, *NA* no activity at a concentration of 200 μg/ml

Discussion

Amphibians are a useful resource in the search for antimicrobial peptides. Many antimicrobial peptides have been identified in amphibian skins. The extreme diversity of such peptides in single amphibian species has been confirmed in several frogs. Two representative cases are *Rana palustris* (Basir et al. 2000), and *O. grahami* (Li et al. 2007). Twenty-two antimicrobial peptides belonging to eight different families were found in skin secretions of *R. palustris* (Basir et al. 2000). A total of 372 cDNA sequences encoding 107 novel antimicrobial peptides belonging to 30 divergent groups were characterized from a single individual skin of the frog *O. grahami* (Li et al. 2007). Recently, antimicrobial peptide diversity has also been demonstrated in the brains of two kinds of *Bombina* toads. A total of 158 cDNA clones encoding 79 antimicrobial peptides belonging to two peptide groups (maximin and maximin-H) were isolated from brain cDNA libraries of *B. maxima* and *B. microdeladigitora* (Liu et al. 2011). The current work identified 31 antimicrobial peptides from the skin of *A. jingdongensis*. Twenty-six of them are novel peptides including two novel antimicrobial peptide groups (Fig. 2). These results confirmed the diversity of antimicrobial peptides in the *Amolops* frog. Most of these antimicrobial peptide families, such as brevinin-1, brevinin-2, odorranain-F, esculentin-2, temporin, amolopin-3, and ranacyclin, are also found in amphibians belonging to the genus *Rana* or *Odorrana*, suggesting a possible evolutionary connection among these three genuses of amphibians.

Table 2 Hemolytic activity of antimicrobial peptides from *A. jingdongensis*

Concentration (μg/ml)	Hemolysis (%)			
	25	50	100	200
Brevinin-1-AJ1	7.62	24.58	83.05	87.65
Temporin-AJ8	1.79	5.22	7.92	10.85
Jingdongin-1	1.32	5.37	6.35	16.55
Jingdongin-2	0.7	1.1	2.6	3.7

Data represent mean values of three independent experiments performed in duplicate

The antimicrobial activity of several antimicrobial peptides from *A. jingdongensis* was tested. It was found that antimicrobial and hemolytic activities of brevinin-1-AJ1 were strong, whereas jingdongin-1 had moderate antimicrobial activity and little hemolytic activity (Tables 1, 2). Brevinin-1-AJ1 and jingdongin-1 have different sequence length, hydrophobicity, and net charge, which may be responsible for their functional difference. The structural and functional comparison between brevinin-1-AJ1 and jingdongin-1 may be instructive for antimicrobial agent design. Jingdongin-1, an anionic peptide containing a rare free half-cysteine, was found to have no antimicrobial activity. It will be interesting to discover what the real function of jingdongin-1 is.

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References

Amiche M, Delfour A, Nicolas P (1998) Opioid peptides from frog skin. EXS 85:57–71

Basir YJ, Knoop FC, Dulka J, Conlon JM (2000) Multiple antimicrobial peptides and peptides related to bradykinin and neuromedin N isolated from skin secretions of the pickerel frog, *Rana palustris*. Biochim Biophys Acta 1543:95–105

Bignami GS (1993) A rapid and sensitive hemolysis neutralization assay for palytoxin. Toxicon 31:817–820

Boman HG (1991) Antibacterial peptides: key components needed in immunity. Cell 65:205–207

Chen Z, Yang X, Liu Z, Zeng L, Lee W, Zhang Y (2012) Two novel families of antimicrobial peptides from skin secretions of the Chinese torrent frog, *Amolops jingdongensis*. Biochimie 94:328–334

Clarke BT (1997) The natural history of amphibian skin secretions, their normal functioning and potential medical applications. Biol Rev Camb Philos Soc 72:365–379

Conlon JM (1999) Bradykinin and its receptors in non-mammalian vertebrates. Regul Pept 79:71–81

Conlon JM, Kolodziejek J, Nowotny N (2004) Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. Biochimica Biophysica Acta 1696(1):1–14

Duda TF Jr, 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, Melchiorri P, Broccardo M, Erspamer GF, Falaschi P, Improota G, Negri L, Renda T (1981) The brain-gut-skin triangle: new peptides. Peptides 2:7–16

Lai R, Zheng YT, Shen JH, Liu GJ, Liu H, Lee WH, Tang SZ, Zhang Y (2002) Antimicrobial peptides from skin secretions of Chinese red belly toad *Bombina maxima*. Peptides 23:427–435

Li J, Liu T, Xu X, Wang X, Wu M, Yang H, Lai R (2006) Amphibian tachykinin precursor. Biochem Biophys Res Commun 350:983–986

Li J, Xu X, Xu C, Zhou W, Zhang K, Yu H, Zhang Y, Zheng Y, Rees HH, Lai R, Yang D, Wu J (2007) Anti-infection peptidomics of amphibian skin. Mol Cell Proteomics 6:882–894

Li J, Wu H, Hong J, Xu X, Yang H, Wu B, Wang Y, Zhu J, Lai R, Jiang X, Lin D, Prescott MC, Rees HH (2008) Odorranalectin is a small peptide lectin with potential for drug delivery and targeting. PLoS One 3:e2381

Liu X, Wang Y, Cheng L, Song Y, Lai R (2007) Isolation and cDNA cloning of cholecystokinin from the skin of *Rana nigrovittata*. Peptides 28:1540–1544

Liu C, Hong J, Yang H, Wu J, Ma D, Li D, Lin D, Lai R (2010) Frog skins keep redox homeostasis by antioxidant peptides with rapid radical scavenging ability. Free Radic Biol Med 48:1173–1181

Liu R, Liu H, Ma Y, Wu J, Yang H, Ye H, Lai R (2011) There are abundant antimicrobial peptides in brains of two kinds of *Bombina* toads. J Proteome Res 10:1806–1815

Lu Y, Li J, Yu H, Xu X, Liang J, Tian Y, Ma D, Lin G, Huang G, Lai R (2006) Two families of antimicrobial peptides with multiple functions from skin of rufous-spotted torrent frog, *Amolops loloensis*. Peptides 27:3085–3091

Severini C, Improta G, Falconieri-Erspamer G, Salvadori S, Erspamer V (2002) The tachykinin peptide family. Pharmacol Rev 54:285–322

Wang Y, Hong J, Liu X, Yang H, Liu R, Wu J, Wang A, Lin D, Lai R (2008a) Snake cathelicidin from *Bungarus fasciatus* is a potent peptide antibiotics. PLoS One 3:e3217

Wang A, Wang J, Hong J, Feng H, Yang H, Yu X, Ma Y, Lai R (2008b) A novel family of antimicrobial peptides from the skin of *Amolops loloensis*. Biochimie 90:863–867

Wang M, Wang Y, Wang A, Song Y, Ma D, Yang H, Ma Y, Lai R (2010) Five novel antimicrobial peptides from skin secretions of the frog *Amolops loloensis*. Comp Biochem Physiol B 155:72–76

Wu J, Liu H, Yang H, Yu H, You D, Ma Y, Ye H, Lai R (2011) Proteomic analysis of skin defensive factors of tree frog *Hyla simplex*. J Proteome Res 10(9):4230–4240

Yan X, Liu H, Yang X, Che Q, Liu R, Yang H, Liu X, You D, Wang A, Li J, Lai R (2011) Bi-functional peptides with both trypsin-inhibitory and antimicrobial activities are frequent defensive molecules in Ranidae amphibian skins. Amino Acids 43:309–316

Yan X, Zhong J, Liu H, Liu C, Zhang K, Lai R (2012) The cathelicidin-like peptide derived from panda genome is a potential antimicrobial peptide. Gene 492:368–374

Yang H, Wang X, Liu X, Wu J, Liu C, Gong W, Zhao Z, Hong J, Lin D, Wang Y, Lai R (2009) Antioxidant peptidomics reveals novel skin antioxidant system. Mol Cell Proteomics 8:571–583

Yasuhara T, Ishikawa O, Nakajima T, Araki K, Tachibana S (1979) The studies on the active peptide on smooth muscle in the skin of *Rana rugosa,* Thr6-bradykinin and its analogous peptide, ranakinin-R. Chem Pharm Bull (Tokyo) 27:486–491

You D, Hong J, Rong M, Yu H, Liang S, Ma Y, Yang H, Wu J, Lin D, Lai R (2009) The first gene-encoded amphibian neurotoxin. J Biol Chem 284(33):22079–22086

Zasloff M (1987) Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc Natl Acad Sci U S A 84:5449–5453

Zasloff M (1992) Antibiotic peptides as mediators of innate immunity. Curr Opin Immunol 4:3–7