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Peptidomic analysis of skin secretions supports separate species status for the tailed frogs, *Ascaphus truei* and *Ascaphus montanus*

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Abstract

The tailed frog *Ascaphus truei* Stejneger, 1899 is the most primitive extant anuran and the sister taxon to the clade of all other living frogs. The species occupies two disjunct ranges in the Northwest region of North America: the Cascade Mountains and coastal area from British Columbia to Northern California, and an inland range in the northern Rocky Mountains and the Blue and Wallowa mountains. A previous study led to the isolation of eight peptides with antimicrobial activity (termed the ascaphins) from skin secretions of *A. truei* from the coastal range. The present study has used peptidomic analysis to identify the products of orthologous ascaphin genes in electrically-stimulated skin secretions from inland range specimens. Structural characterization of the peptides demonstrated that ascaphins from the inland range contained the following amino acid substitutions compared with orthologs from the coastal range frogs: ascaphin-1 (Ala¹² → Glu), ascaphin-3 (Asp⁴ → Glu), ascaphin-4 (Ala¹⁹ → Ser), ascaphin-5 (Lys¹² → Thr), and ascaphin-7 (Gly⁸ → Ser and Ser²⁰ → Asn). Orthologs of ascaphins-2, -6, and -8 were not identified but a paralog of ascaphin-5, identical to ascaphin-5 from coastal range frogs, was found. The data support the claims, derived from analysis of the nucleotide sequences of mitochondrial genes, that the inland populations of the tailed frog should be recognized as a distinct species, the Rocky Mountain tailed frog *Ascaphus montanus* and that the divergence of the species from *A. truei* probably occurred in the late Miocene (approximately 10 Mya).

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1. Introduction

Our understanding of the evolutionary history of Anura (frogs and toads) is currently in a considerable state of flux. The application of molecular techniques of phylogenetic analysis, particularly the comparison of nucleotide sequences of orthologous mitochondrial genes, has led to a reappraisal of many taxonomic classifications that had been established using morphological criteria (Hillis and Wilcox, 2005; Frost et al., 2006). Cationic α -helical peptides with broad-spectrum antibacterial and antifungal activities are synthesized in the skins of

many, but by no means all, species of anurans and represent a component of the organism's system of innate immunity that defends it against invasion by pathogenic microorganisms (Nicolas and Mor, 1995). On the basis of common structural features, these antimicrobial peptides may be grouped together in families that share a common evolutionary origin but the variation in amino acid sequences of homologous peptides is considerable (Simmaco et al., 1998; Conlon et al., 2004a). It is rare that orthologs from two different species have an identical amino acid sequence, even when they are quite closely related phylogenetically. Consequently, determination of the primary structures of the dermal peptides can be used to complement morphological and other types of molecular analysis in gaining insight into taxonomic and phylogenetic relationships of anurans.

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The tailed frog *Ascaphus truei* occupies a uniquely important position in anuran phylogeny as the most primitive extant frog that may be regarded as the sister group to the clade of all other living frogs (Ford and Cannatella, 1993; Roelants and Bossuyt, 2005; Gissi et al., 2006). It retains many morphological features that have been lost in all other extant anuran lineages [reviewed in (Nielson et al., 2001)] and is the only North American frog that reproduces by internal fertilization. It inhabits cold, forested streams and has evolved several adaptive features to survive in this environment (Metter, 1964). *Ascaphus* occupies two disjunct ranges in the northwest region of North America — the Cascade Mountains and coastal region from British Columbia south to Northern California, and an inland range in the northern Rocky Mountains and the Blue and Wallowa mountains (Nussbaum et al., 1983). The ranges are well separated by a zone of reduced rainfall. The coastal and inland groups may be distinguished on the basis of morphological characters (Pauken and Metter, 1971) but they were traditionally regarded as a single species. It was postulated that populations were connected by gene flow through the highlands of central Oregon until the close of the Pleistocene (approximately 12,000 years BP) (Metter and Pauken, 1969; Pauken and Metter, 1971). More recently, however, analysis based upon the comparisons of nucleotide sequences of mitochondrial cytochrome *b* and NADH dehydrogenase genes from specimens inhabiting different sites throughout the coastal and inland ranges has suggested that the divergence of the two groups is more ancient and occurred in response to the rise of Cascade Mountains during the late Miocene (approx. 10 million years Mya) (Nielson et al., 2001; Carstens et al., 2005; Nielson et al., 2006). Although there is significant genetic variability within each population, frogs from the coastal and inland ranges form two well differentiated clades leading to the proposal that animals from the inland range should be regarded as a separate species, the Rocky Mountain tailed frog *A. montanus* (Nielson et al., 2001).

A previous study led to the isolation and structural characterization of eight structurally-related, cationic α -helical peptides, designated ascaphins 1–8, from skin secretions taken from coastal range specimens collected in Humboldt County, northern California (Conlon et al., 2004b). These peptides display broad-spectrum antimicrobial activity and varying degrees of cytolytic activity against human erythrocytes. The aim of the present study was to use the same methodology to characterize orthologous ascaphins present in skin secretions of specimens from the inland range. Determination of the degree of variability in the amino acid sequences of the ascaphins permits critical appraisal of the proposal that the two populations should be regarded as separate species and provides insight into when the populations may have diverged.

2. Materials and methods

2.1. Collection of skin secretions

Adult male specimens of *Ascaphus* ($n=2$; length 4.1 and 4.8 cm, body wt. 5.3 and 6.8 g respectively) were collected

under permit (GLAC-2005-SCI-0040) in October, 2005 from Fern Creek, Glacier National Park, Montana, USA. Electrically-stimulated skin secretions were collected as previously described (Conlon et al., 2005a) and immediately frozen on dry ice for shipment to U.A.E. University. The secretions from each animal were pooled and passed at a flow rate of 2 mL/min through 4 Sep-Pak C-18 cartridges (Waters Associates) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70.0:29.9:0.1, v/v/v) and freeze-dried.

2.2. Peptide purification

The lyophilized skin secretions, after partial purification on Sep-Pak cartridges, were redissolved in 0.1% (v/v) trifluoroacetic acid/water (2 mL) and injected onto a (1.0 \times 25-cm) Vydac 218TP510 (C-18) reverse-phase HPLC column (Separations Group) equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 2.0 mL/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 49% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 nm and 280 nm and fractions (1 min) were collected.

Aliquots of fractions from the major uv-absorbing peaks with retention times between 40 and 70 min (peaks 1–11 in Fig. 1) were analyzed by MALDI–TOF mass spectrometry 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 2000–4000 Da range. The accuracy of mass determinations was $\pm 0.02\%$. Peaks 2, 4, 5, 6, and 7 (containing the ascaphins) were rechromatographed on a (0.46 \times 25-cm) Vydac 214TP54 (C-4) column. The concentration of acetonitrile in the eluting solvent was raised from 21% to 49% over 50 min and the flow rate was 1.5 mL/min.

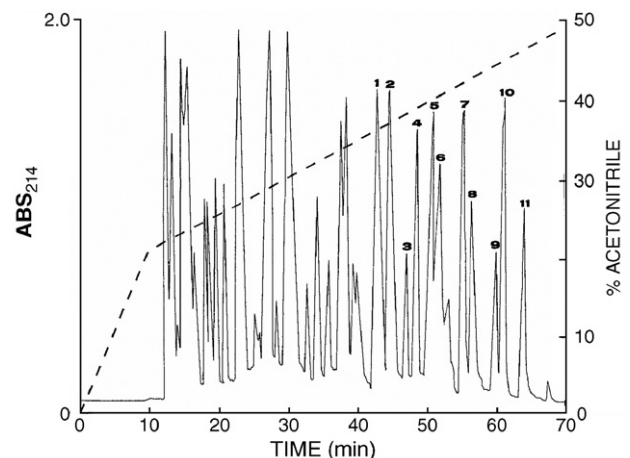


Fig. 1. Reversed-phase HPLC on a semipreparative Vydac C-18 column of skin secretions from *Ascaphus montanus*, after partial purification on Sep-Pak cartridges. Peptides in the peaks designated 1–11 were analysed by MALDI–TOF mass spectrometry. Peaks 2, 4, 5, 6, and 7 contained ascaphins and were subjected to further purification. The dashed line shows the concentration of acetonitrile in the eluting solvent.

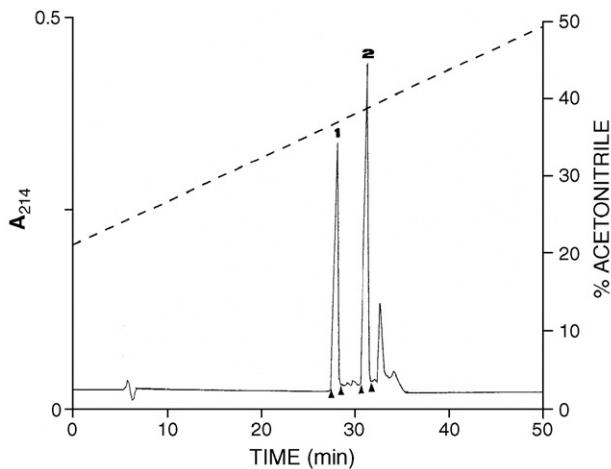


Fig. 2. Separation of ascaphin-3M (peak 1) and ascaphin-5Ma (peak 2) on an analytical Vydac C-4 column. The arrowheads show where peak collection began and ended.

2.3. Structural characterization

The primary structures of the ascaphins were determined by automated Edman degradation using a model 494 Procise sequenator (Applied Biosystems). Amino acid composition analyses were performed by the University of Nebraska Medical Center Protein Structure Core Facility (Omaha, NE, USA).

3. Results

3.1. Purification of the peptides

The skin secretions, after concentration and partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 semipreparative reverse-phase HPLC column (Fig. 1). Analysis by mass spectrometry of the major peaks in the chromatogram eluting between 40 and 70 min indicated the following distribution of molecular masses (a.m.u.): peak 1, 1198; peak 2, 2426; peak 3, no signal; peak 4, 2601; peak 5, 2496+2590, peak 6, 2589; peak 7 2630; peak 8, 1453; peak 9, 1292; peak 10, 1306; peak 11, 1476. The peaks containing the ascaphins (peptides with masses in the range 2000–3000) were selected for further purification by chromatography on an analytical Vydac C-4 column. The ascaphins from the inland range animals are designated M to differentiate them from the

		M_r (obs)	M_r (calc)
Ascaphin-1M	GFRDVLKGAAKEFVKTVAGHIAN.NH ₂	2426.3	2426.4
Ascaphin-3M	GPREV LKGAAKAFVKTVAGHIANI	2496.5	2496.4
Ascaphin-4M	GPKDWIKGAAKKLIKTVASNIANQ	2600.5	2600.5
Ascaphin-5Ma	GIKDWIKGAAKKLIKTVASHIANQ	2589.9	2589.5
Ascaphin-5Mb	GIKDWIKGAARKLIKTVASHIANQ	2562.5	2562.5
Ascaphin-7M	GPKDWIKSAAKLIKTVASNIANQ	2630.4	2630.5

Fig. 3. Amino acid sequences, observed molecular masses (M_r obs), and calculated molecular masses (M_r calc) of ascaphins isolated from skin secretions of *A. montanus*.

Ascaphin-1	GFRDVLKGAAKAFVKTVAGHIAN
Ascaphin-1M	-----E-----
Ascaphin-3	GFRDVLKGAAKAFVKTVAGHIANI
Ascaphin-3M	---E-----
Ascaphin-4	GPKDWIKGAAKKLIKTVASNIANQ
Ascaphin-4M	-----S-----
Ascaphin-5	GIKDWIKGAAKKLIKTVASHIANQ
Ascaphin-5Ma	-----T-----
Ascaphin-5Mb	-----T-----
Ascaphin-7	GPKDWIKGAAKKLIKTVASSIANQ
Ascaphin-7M	-----S-----N-----

Fig. 4. A comparison of the primary structures of peptides belonging to the ascaphin family from *A. montanus* and *A. truei*. (–) denotes those amino acids that are conserved in the peptides.

ascaphins from the coastal range and isoforms are identified by lower case letters. The methodology is illustrated by the separation of ascaphin-3M and ascaphin-5Ma (Fig. 2). All peptides were purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry. The final yields of purified peptides (nmol), estimated from the integrated area under the peaks, were: ascaphin-1M, 45; ascaphin-3M, 21; ascaphin-4M, 42; ascaphin-5Ma, 26; ascaphin-5Mb, 18; and ascaphin-7, 13.

3.2. Structural characterization

The primary structures of the ascaphins were established by automated Edman degradation and their amino acid sequences are shown in Fig. 3. The observed molecular masses of the peptides, determined by MALDI–TOF mass spectrometry, are consistent with the proposed sequences (Fig. 3). It was not possible to determine the C-terminal amino acid residue in ascaphin-3M but amino acid composition analysis demonstrated that this residue was isoleucine (Found: Asx 1.0 (1), Glx 1.0 (1), Gly 3.0 (3), His 0.9 (1), Arg 1.0 (1), Thr 1.0 (1), Ala 5.0 (5), Val 2.8 (3), Ile 1.8 (2), Leu 1.0 (1), Lys 2.8 (3) residues/mol peptide). The figures in parentheses show the number of residues predicted from the proposed sequence.

<u>Brevinin-1</u>	
<i>R. draytonii</i>	FLPILAGLAAKIVPKVFCLITKKC
<i>R. draytonii</i>	-----T-----
<i>R. draytonii</i>	-----V-----
<i>R. draytonii</i>	-----DML-----S-----
<i>R. aurora</i>	-----L-----S-----
<i>R. aurora</i>	-----N-L-----S-----
<u>Ranatuerin-2</u>	
<i>R. draytonii</i>	GIMDTFGVAKGVAKDLAVKLLDNFKCKITGC
<i>R. draytonii</i>	-----I-----N--G---EL--M---
<i>R. aurora</i>	--LSS-----N--G---EL-----

Fig. 5. A comparison of the primary structures of peptides belonging to the brevinin-1 and ranatuerin-2 families isolated from skin secretions of *Rana aurora* and *Rana draytonii*. (–) denotes those amino acids that are conserved in the peptides.

4. Discussion

The members of the ascaphin family of antimicrobial peptides are structurally similar to each other, suggesting an origin that involves multiple duplications of an ancestral gene (Duda et al., 2002), but they do not resemble closely any antimicrobial peptide isolated from the skins of other frog species (Conlon et al., 2004b). The ascaphins do, however, display limited sequence similarity with the cationic, amphipathic α -helical peptides pandinin 1 (Corzo et al., 2001) and opistoporin 1 (Moerman et al., 2002) isolated from the venoms of African scorpions. The primary structures of the ascaphins isolated from tailed frogs from the inland range (designated M) are compared with the previously isolated products of orthologous genes from specimens from coastal range in Fig. 4. The following amino acid substitutions were found: ascaphin-1 (Ala¹² → Glu), ascaphin-3 (Asp⁴ → Glu), ascaphin-4 (Ala¹⁹ → Ser), ascaphin-5 (Lys¹² → Thr), and ascaphin-7 (Gly⁸ → Ser and Ser²⁰ → Asn). Orthologs of ascaphin-2 and -8 were not detected in the secretions from the inland range frogs. Ascaphin-6 was also not detected but this peptide contains the single substitution Gln²⁴ → Glu compared with ascaphin-7 and thus may represent an artifactually modified form of ascaphin-7 arising from hydrolysis during the purification procedure. The differences in primary structures among the ascaphins are sufficiently great to lend support to the assertion that the inland range and coastal range specimens of the tailed frog should be regarded as separate species (Nielson et al., 2001).

The present study complements a previous investigation (Conlon et al., 2006) in which peptidomic analysis of skin secretions supported the claim, based upon mitochondrial DNA analysis (Shaffer et al., 2004), that the Northern red-legged frog *Rana aurora aurora* and the California red-legged frog *Rana aurora draytonii*, should be regarded as separate species rather than classified together in the same species group. The red-legged frogs are combined with *R. boylei*, *R. muscosa*, *R. cascadae*, *R. pretiosa*, and *R. luteiventris* in the *Amerana* species group (Hillis and Wilcox, 2005). This assemblage forms a well-defined monophyletic clade that is approximately 8 million years old (Macey et al., 2001). As shown in Fig. 5, the peptides of the brevinin-1 family and the ranatuerin-2 family isolated from *R. aurora* and *R. draytonii* show a comparable, but somewhat greater, degree of variability as the orthologous ascaphins from *A. truei* and *A. montanus*. While the divergence of *R. aurora* and *R. draytonii* probably occurred later than that of *Ascaphus*, it is possible that the long generation time (up to 6–8 years) (Daugherty and Sheldon, 1982) and adaptation to low temperatures of *Ascaphus* may result in a slower evolutionary rate and reduced genetic divergence compared with frogs of the genus *Rana* (Martin and Palumbi, 1993; Rand, 1994). In this light, the data from the present study indicate that a divergence of the populations in the late Miocene (approximately 10 Mya) (Nielson et al., 2001; Nielson et al., 2006) is much more probable than at the close of the Pleistocene (approximately 12,000 years BP) (Metter and Pauken, 1969). Molecular analysis has revealed similar deep divergences between coastal and inland populations of other amphibian lineages in the Pacific Northwest (*Dicamp-*

ton spp., and between *Plethodon vandykei* and *P. idahoensis*) (Carstens et al., 2005).

The limited amount of purified peptides isolated from *A. montanus* in this study did not permit an investigation of their antimicrobial potencies or toxicities towards eukaryotic cells. The cytolytic activity of a frog skin peptide against microorganisms is determined by a complex interaction involving cationicity, hydrophobicity, α -helicity and amphipathicity (Yeaman and Yount, 2003). The bacterial cytoplasmic cell membrane is rich in anionic phospholipids, such as phosphatidylglycerol, and negatively charged lipopolysaccharides so that a decrease in peptide cationicity should weaken interaction with the negatively charged bacterial cell membrane and decrease antimicrobial potency. Consequently, the substitutions (Ala¹² → Glu) in ascaphin-1M and (Lys¹² → Thr) in ascaphin-5Mb would be expected to decrease antimicrobial potency significantly. Skin secretions, as well as protecting amphibians against pathogenic microorganisms, can be both toxic and distasteful to predators. Little is known about the defense strategy of *Ascaphus* but its low temperature aquatic environment is probably not conducive to the growth of microorganisms and so it is tempting to speculate that the primary role of the ascaphins is to deter ingestion by predators, such as snakes and birds. It has been shown previously that skin secretions from coastal range *A. truei* contain multiple bradykinin-related peptides that may serve the same purpose (Conlon et al., 2005a,b).

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