

Selection for Antimicrobial Peptide Diversity in Frogs Leads to Gene Duplication and Low Allelic Variation

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Abstract Antimicrobial peptides are highly diverse pathogen-killing molecules. In many taxa, their evolution is characterized by positive selection and frequent gene duplication. It has been proposed that genes encoding antimicrobial peptides might be subject to balancing selection and/or an enhanced mutation rate, but these hypotheses have not been well evaluated because allelic variation has rarely been studied at antimicrobial peptide loci. We present an evolutionary analysis of novel antimicrobial peptide genes from leopard frogs, *Rana*. Our results demonstrate that a single genome contains multiple homologous copies, among which there is an excess of nonsynonymous nucleotide site divergence relative to that expected from synonymous site divergence. Thus, we confirm the trends of recurrent duplication and positive selection. Allelic variation is quite low relative to interspecies divergence, indicating a recent positive selective sweep with no evidence of balancing selection. Repeated gene duplication, rather than a balanced maintenance of divergent allelic variants at individual loci, appears to be how frogs have responded to selection for a diverse suite of antimicrobial peptides. Our data also support a pattern of enhanced synonymous site substitution in the mature peptide region of the gene, but we cannot conclude that this is due to an elevated mutation rate.

Keywords Antimicrobial peptide · Gene duplication · Positive selection · Allelic variation · Mutation rate · d_N/d_S · HKA test · *Rana pipiens* · *Rana chiricahuensis*

Introduction

Unlike much of the genome, genes of the immune system are known to frequently evolve non-neutrally (Garrigan and Hedrick 2003). Thus, they permit the study of natural selection and adaptation at its fundamental molecular level. Studying immunity gene evolution is also important because changes driven by positive selection are likely to convey novel functional properties, and functionally divergent disease resistance genes could lead to distinct practical therapeutic applications. Furthermore, it is particularly critical to understand how hosts adapt to pathogens, because emerging infectious diseases are exerting strong novel selective pressures on many species (Daszak et al. 2000; Altizer et al. 2003).

Antimicrobial peptides are short, cationic, amphipathic molecules that kill bacteria, viruses, and fungi through a noncatalytic disruption of the microbial membrane (Yeaman and Yount 2003; Tennessen 2005a). The mature peptide is often cleaved off a larger protein containing a signal sequence and a propeptide (Tennessen 2005a). These molecules have been studied in many taxa. In ranid frogs, antimicrobial peptides include the brevinins and the ranatuerins, which consist of an α -helix with a conserved disulfide bridge forming a six- or seven-residue loop known as the “Rana box” at the C-terminal end (Park et al. 1994). Because they are easily obtained from the skin of live specimens, a large number of ranid frog antimicrobial peptides have been described (Conlon et al. 2004). They are diverse in functional properties, and many are active against human microbes and/or against pathogens causing emerging infectious diseases in amphibians (Rollins-Smith et al. 2002; Chinchar et al. 2004; Rollins-Smith and Conlon 2005). Much less is known about the genes encoding these peptides.

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In this study, we sequence brevinin and ranatuerin loci from three closely related species: the northern leopard frog, *Rana pipiens*; the southern leopard frog, *R. sphenoccephala*; and the Chiricahua leopard frog, *R. chiricahuensis* (Hillis and Wilcox 2005). We address four principal questions.

Has frequent gene duplication resulted in multiple copies of antimicrobial peptide genes within leopard frog genomes?

All animal species appear to carry multiple duplicated copies of antimicrobial peptide genes, with vertebrate genomes typically containing tens of loci (Schutte et al. 2002; Lynn et al. 2004). In most taxa that have been examined, several rounds of duplication have occurred relatively recently (Maxwell et al. 2003; Semple et al. 2003; Bulmer and Crozier 2004; Tennessen 2005a). Gene copy number has not been well investigated in amphibians, though. Several similar peptides can often be isolated from the same frog species, suggesting multiple loci (Conlon et al. 2004). A suite of unique antimicrobial peptides would presumably maximize a host's ability to fight disease, and thus we hypothesize that selection has favored frequent duplication of these genes in leopard frogs.

Has positive selection driven the evolution of leopard frog antimicrobial peptides?

Positive natural selection on antimicrobial peptide genes occurs often in many animal taxa (Tennessen 2005a). The primary evidence for positive selection is that the ratio of the rate of nonsynonymous substitution per nonsynonymous nucleotide site to the rate of synonymous substitution per synonymous nucleotide site, or d_N/d_S , frequently exceeds one. The few available sequences of frog antimicrobial peptide genes suggest that these genes also evolve via positive natural selection, at least in some cases (Duda et al. 2002). Thus, antimicrobial peptides are potentially useful models for studying non-neutral molecular evolution. By testing the hypothesis that positive selection has occurred among the leopard frog sequences we examine, we add to the overall understanding of when and how antimicrobial peptides evolve adaptively. For example, does positive selection only occur immediately after gene duplication, or are there also adaptive differences between orthologs? Peptides that have diverged due to positive selection are also important to identify because they are more likely to differ in their functional properties.

Is allelic variation at antimicrobial peptide loci affected by non-neutral evolution?

Allelic variation at antimicrobial peptide loci has never been systematically studied in a nonhuman vertebrate. Adaptive genetic variation is often high in other types of immunity genes, including the major histocompatibility complex (MHC) of vertebrates (Garrigan and Hedrick 2003; Piertney and Oliver 2006) and plant R-genes (Bergelson et al. 2001; Bakker et al. 2006). Multiple variants of antimicrobial peptides would presumably provide an adaptive benefit, so it is possible that balancing selection maintains several alleles at individual loci via overdominance, frequency-dependent selection, and/or geographically heterogeneous selection due to distinct microbial communities affecting different populations (Tennessen 2005a). Alternatively, generalist immunity genes like antimicrobial peptide loci might harbor less diversity than specialist immunity genes (Tiffin et al. 2004). Under this scenario, recurrent gene duplication might satisfy the need for multiple antimicrobial peptide variants, while selective sweeps reduce allelic variation at individual loci. Distinct peptides are sometime found in frogs of the same species from different geographic locations (e.g., Won et al. 2004), but it is unclear whether these represent allelic variants or whether such variation is selectively neutral. We examine loci for allelic diversity, and we test whether variation and divergence at a particular locus in *R. pipiens* differs from neutral expectations by comparing it with other nuclear loci.

To what extent is the synonymous substitution rate in the mature peptide region enhanced, and why?

Another widespread trend in antimicrobial peptide genes is a greater degree of synonymous site divergence in the mature peptide region, relative to the rest of the gene (Vanhoey et al. 2003; Tennessen 2005b). In frogs, this pattern has been shown with respect to the signal sequence, but it is unknown whether it holds with respect to noncoding sequence such as introns. The two most likely explanations for this phenomenon are an elevated mutation rate or natural selection acting on supposedly silent sites. The hypothesis of an elevated mutation rate predicts an excess of allelic variation relative to other loci. Because allelic variation is also influenced by natural selection, it cannot be used as a direct estimate of the relative mutation rate, but it can provide suggestive evidence as to whether the mutation rate is abnormally high.

Materials and Methods

Samples

Specimens of *R. pipiens* and *R. sphenoccephala* were collected from multiple populations as described previously (Hoffman and Blouin 2004). Specimens of *R. chiricahuensis* consisted of toe clips collected September 2001 in Arizona, USA, and preserved by desiccation in 1.5 ml tubes filled with Drierite desiccant (W. A. Hammond Drierite Co., Xenia, OH). DNA was extracted as described previously (Hoffman and Blouin 2004).

Most polymerase chain reactions (PCRs) were carried out as 25 μ l reactions using standard buffer conditions, 1.5 mM MgCl₂, 0.2 mM each deoxyribonucleotide triphosphate, approximately 100 ng DNA, and 0.5 Units *Taq* DNA polymerase. The exceptions, noted below, employed either *Pfu* DNA polymerase (Promega, Madison, WI) or the TaqPlus Long PCR System (Stratagene, La Jolla, CA). For all sequences, the PCR product was visualized under ultraviolet light, purified with the MoBio Ultraclean PCR cleanup kit (Salina Beach, CA), and sequenced by the Nevada Genomics Center (Reno, NV).

Brevinin Loci

In order to estimate the minimum number of brevinin loci, to test for evidence of positive selection via the d_N/d_S ratio, and to compare synonymous site divergence with non-coding divergence, we cloned and sequenced brevinin genes. We designed primers for brevinin-1Pb (Brev1PF1: 5'-TCC TTC TAC TCC TTT TCT TC-3'; Brev1PF2: 5'-CAT CAA CTT ATC TTT CTG TG-3'; Brev1PR1: 5'-CCA ATT CAA GTT TCC AAA G-3'; Brev1PR2: 5'-TTT CAT CTG GCT CAT CTA TTC-3') using an available cDNA sequence (Accession AJ427746; Chen et al. 2003). We amplified brevinin-1Pb from genomic *R. pipiens* DNA with the primers Brev1PF1 and Brev1PR1 using the TaqPlus Long PCR System under the following conditions: an initial denaturation step at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 51°C for 30 sec, and extension at 72°C for 8 min; and a final extension step at 72°C for 5 min. We then amplified the resultant PCR product with the nested primers Brev1PF2 and Brev1PR2 using the TaqPlus Long PCR System under the following conditions: an initial denaturation step at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 54°C for 30 sec, and extension at 72°C for 5.5 min; and a final extension step at 72°C for 30 min. We partially sequenced the resultant PCR product from the 3' end and used it to design the primer Brev1PF3 (5'-TTA GAA CAA TAT TAG ATG ACC-3').

We used the primers Brev1PF3 and Brev1PR1 in PCR reactions with the high-fidelity enzyme *Pfu* DNA polymerase and genomic DNA template from each of the following frogs: two *R. pipiens* individuals from the same population in Ontario, Canada (RpO1 and RpO2), a *R. pipiens* individual from British Columbia, Canada (RpBC1), and a *R. chiricahuensis* individual (Rc1). PCR amplification conditions consisted of an initial denaturation step at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 54°C for 30 sec, and extension at 72°C for 1 min; and a final single extension step at 72°C for 5 min. The resultant PCR products were incubated with *Taq* polymerase in order to add 3' adenines and cloned into *E. coli* using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). The recombinant locus was amplified from screened colonies using the primers T3 and T7 under the following PCR conditions: an initial denaturation step at 94°C for 3 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min; and a final single extension step at 72°C for 7 min. The resultant PCR products were sequenced with either T3 or T7. In addition, we used the primers Brev1PF3 and Brev1PR1 to amplify genomic DNA from two *R. sphenoccephala* individuals under the aforementioned PCR conditions, and the resultant PCR products were sequenced without cloning.

Ranatuering Loci

In order to estimate the minimum number of ranatuering loci and to test for evidence of positive selection with the d_N/d_S ratio, we sequenced ranatuering genes. We designed primers for the ranatuering-2P gene (Ranat2PF1: 5'-ATG AAG AAA TCC CTG TTA CTC-3'; Ranat2PF2: 5'-ATC TCC TTA TCT CTC TGT G-3'; Ranat2PR1: 5'-CAC ATC AGA TGA CTT CCA ATT-3'; Ranat2PR2: 5'-TTC CAC ACC ATC GTC TCC-3') using an available cDNA sequence (Accession AJ427747; Chen et al. 2003). We amplified the ranatuering-2P gene from genomic *R. pipiens* DNA with the primers Ranat2PF1 and Ranat2PR1 using the TaqPlus Long PCR System under the following conditions: an initial denaturation step at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 53°C for 30 sec, and extension at 72°C for 8 min; and a final extension step at 72°C for 30 min. We then amplified the resultant PCR product with the nested primers Ranat2PF2 and Ranat2PR2 using the TaqPlus Long PCR System under the following conditions: an initial denaturation step at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 30 sec, and extension at 72°C for 5.5 min; and a final extension step at 72°C for 30 min. We partially sequenced the resultant PCR product and used it

to design the primer Ranat2PF3 (5'-TCA ATG TTG TTT TAT GTA AGG-3'). We also designed the primer Ranat2PF4 (5'-AAG CCG ATG ACG ACC AAG-3') using an available cDNA sequence (AJ427748; Chen et al. 2003).

We used the primers Ranat2PF3 and Ranat2PR1 to amplify genomic DNA from *R. pipiens* and *R. chiricahuensis*. PCR amplification conditions consisted of an initial denaturation step at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 54°C for 30 sec, and extension at 72°C for 1 min; and a final single extension step at 72°C for 5 min. The resultant PCR product was sequenced. In addition, we used the primers Ranat2PF4 and Ranat2PR1 to amplify genomic DNA from *R. pipiens* and *R. chiricahuensis*. PCR amplification conditions consisted of an initial denaturation step at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec; and a final extension step at 72°C for 5 min. The resultant PCR product was sequenced.

In order to test for evidence of balancing selection and an enhanced mutation rate, we examined allelic variation at two particular ranatuerin loci. We amplified genomic DNA from 24 specimens of *R. pipiens* and 4 specimens of *R. chiricahuensis* with the primers Ranat2PF3 and Ranat2PR1. Each *R. pipiens* specimen was collected from a unique population from 1 of 13 states and provinces across North America, representing the major phylogeographic groups of this species as determined from mitochondrial DNA (Hoffman and Blouin 2004). We also amplified genomic DNA from three specimens of *R. pipiens* and four specimens of *R. chiricahuensis* with the primers Ranat2PF4 and Ranat2PR1. The resultant PCR products were sequenced.

For the purpose of an Hudson–Kreitman–Aguadé (HKA) test (Hudson et al. 1987), we compared the gene coding for ranatuerin-2P with two putatively neutral loci coding for arcadlin and type-1 myosin heavy chain. Arcadlin is a cadherin involved in synaptic reorganization (Crump et al. 2002), and myosin is a muscle fiber protein (Lutz et al. 1998). We designed primers for the arcadlin gene (ArcadF: 5'-AAC AGA AGC AGT CAC-3'; ArcadR: 5'-CGC TGT CAT TGA AGT CAC-3') using an available cDNA sequence (Accession AF279872; Crump et al. 2002). We also designed primers for the myosin gene (MyosinF: 5'-GAA GAA CCT TGA ACA GAC-3'; MyosinR: 5'-ATT CTT CTT GTC CTC CTC-3') using an available cDNA sequence (Accession AF013132; Lutz et al. 1998). We amplified genomic DNA from 13 *R. pipiens* individuals and 2 *R. chiricahuensis* individuals under the following conditions for both arcadlin and myosin primer pairs: an initial denaturation step at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 52°C for 30 sec, and extension at 72°C for 1 min; and a

final extension step at 72°C for 5 min. The resultant PCR products were sequenced. To determine whether adaptive substitutions occurred in the *R. pipiens* or the *R. chiricahuensis* lineage, we inferred the common ancestral sequences for the genes coding for ranatuerin-2P, arcadlin, and myosin. Ancestral state reconstruction for the ranatuerin-2P gene employed published DNA (Accession AM113511, AM113509; Chen et al. 2006) and amino acid (Fig. 1) sequences from closely related outgroup species, while ancestral state reconstruction for the arcadlin and myosin genes employed sequences that we amplified from *R. sphenoccephala*.

Analysis

All sequences have been deposited in GenBank under accession numbers DQ276956–DQ276977 and DQ923137–DQ923159. Heterozygotes were identified by the presence of double peaks. Evolutionary analyses were performed with PAML (vers. 3.13a, Yang 1997), DnaSP (vers. 4.0, Rozas et al. 2003), and PAUP* (vers. 4.0b10, Swofford 2002). We used the *maxchi* method (Maynard Smith 1992) to test for recombination, which could invalidate any phylogeny-based tests of selection. To test for positive selection with PAML, we ran two models: one in which d_N/d_S is estimated from the data, and one in which d_N/d_S is constrained to be one. If the estimated value of d_N/d_S is greater than one, and this model is significantly better than the constrained model in a log-likelihood test, we can conclude that positive selection has occurred. We did not use any PAML models that allow d_N/d_S to differ among codons or among branches. Although conserved regions indicate that brevinin and ranatuerin genes are homologous (Vanhoey et al. 2003), the mature peptide regions are too divergent to be aligned reliably, so we analyzed these two gene families separately.

Results

Brevinin Loci

Our initial brevinin locus amplification resulted in a PCR product of approximately 5,000 base pairs. This was partially sequenced to reveal part of an intron, followed by the C-terminal region of the propiece, immediately followed by the mature peptide region (Fig. 1). We designed a forward primer that annealed in the intron, Brev1PF3. Using Brev1PF3 and Brev1PR1, we obtained 227 cloned brevinin-1 sequences consisting of 63 unique haplotypes among the frogs RpO1 (110 sequences, 35 haplotypes), RpO2 (43 sequences, 9 haplotypes), RpBC1 (42 sequences, 10

Fig. 1 Amino acid sequences encoded by the antimicrobial peptide genes observed in this study, aligned with related peptides from the genus *Rana* (Conlon et al. 1999, 2003; Basir et al. 2000; Goraya et al. 2000; Ali et al. 2002; Chen et al. 2003, 2006). The names of previously undescribed mature peptides are presented in bold. A single exon encodes the C-terminal end of the propiece and the mature peptide region, and this is immediately preceded by an intron, which we also partially sequenced in most cases. Ranatuerin-2STa is a reconstructed extinct peptide from the most recent common ancestor of *R. pipiens* and *R. chiricahuensis*

Brevinins

propiece (partial)	mature peptide
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This study:

NAEEE-RRDEPDETDVEVEKRFPLPIAGVAAKVFPPKIFCAISKKC
S.G.....I.....
S.....I.....FL.....
P.V...GQ.LK...T.....
G.GK---.ETGI.LLP.L.NLCRP.Y.T.T.N.
G.GK---.ETGI.LLP.L.NLCRP.-----N.
M.....L.L.L...T.....
M.....V.L.L.L.L...T.....
R.G..EM---.QPGL.LLT.L.NLLRP.Y.T.TQN.
S.G.....A.V.A..FL.....
S.G.....F.A.AI...F.....T.....

Others:

.....S.....S.....
S.....N.S.....
A.V.A.GQFL.....
F.V...GQ.LK..Y.T.....
A.....FL.....
L.L.NFL...T.....
V.....FL...T.....

Peptide name: Species:

Brevinin-1Pa *R. pipiens*
 Brevinin-1Pa *R. pipiens*
 Brevinin-1Pb *R. pipiens*
 Brevinin-1Pe *R. pipiens*
Brevinin-1Pf *R. pipiens*
Brevinin-1Pg *R. pipiens*
Brevinin-1Ph *R. pipiens*
Brevinin-1Pi *R. pipiens*
Brevinin-1CHa *R. chiricahuensis*
Brevinin-1CHb *R. chiricahuensis*
Brevinin-1CHc *R. chiricahuensis*
 Brevinin-1Sb *R. sphenocephala*
Brevinin-1Sd *R. sphenocephala*

 Brevinin-1Pc *R. pipiens*
 Brevinin-1Pd *R. pipiens*
 Brevinin-1Sa *R. sphenocephala*
 Brevinin-1Sc *R. sphenocephala*
 Brevinin-1Bd *R. berlandieri*
 Brevinin-1PLb *R. palustris*
 Brevinin-1PLc *R. palustris*

Ranatuerins

propiece (partial)	mature peptide
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This study:

RGAEDDGVEITEEBEVKRLGLMDTVKNVAKN---LAGHMLDKLKCKITG-C
A.....QL..R.....
A.....QL.....
 ..D.Q.-.V-QQ...SPLT...KLVT.LAA...TVI.TI...V..G.RT
 ..D.QV-.V-QQ...FLS...L.T...V..TVI.T...V..G.RT

Others:

..D.Q.-.V-QQ...FLS...L.T...V..TVI.TI...V..G.RK
A.....QL..TI...M...
 ..I.....A.....QL.....A...
 ..I.....A..D...QL.....R.....
 ..I..S.....I..QL.....
 ..I..S..GL.....KL..S.....
 ..L..I..T...VGL...I..M...
 ..I.....G...T...V.ASL.....

Ranatuerin-2P *R. pipiens*
Ranatuerin-2CHa *R. chiricahuensis*
Ranatuerin-2STa Ancestral
Ranatuerin-2Pb *R. pipiens*
Ranatuerin-2CHb *R. chiricahuensis*

 Ranatuerin-2Pa *R. pipiens*
 Ranatuerin-2ARa *R. areolata*
 Ranatuerin-2PLa *R. palustris*
 Ranatuerin-2PLb *R. palustris*
 Ranatuerin-2PLd *R. palustris*
 Ranatuerin-2BYb *R. boyllii*
 Ranatuerin-2Va *R. versabilis*
 Ranatuerin-2Vb *R. versabilis*

haplotypes), and Rc1 (32 sequences, 12 haplotypes). However, many of these haplotypes likely represent PCR-induced errors such as point mutation or recombination (Cronn et al. 2002), which can occur even with high-fidelity polymerase. Of the 63 haplotypes, 42 were only observed once, and 1 was only observed twice; out of these 43, 24 could have been formed by a single recombination event between two other multiply observed haplotypes in that same individual. To minimize the possibility of PCR-induced errors and to conservatively estimate the number of brevinin-1 loci, we excluded from further analysis all haplotypes observed only once or twice. Our final dataset of cloned brevinin-1 genes thus consisted of 20 unique haplotypes of 438 to 461 nucleotides, all of which were observed at least three times in the same individual. Nine were found in RpO1, five were found in RpOC2, four were found in RpBC1, and five were found in Rc1 (Fig. 2). We also directly sequenced 222 nucleotides of two additional brevinin-1 genes from *R. sphenocephala*. The 22 haplotypes from the three species each contained a single exon and encoded a total of 13 unique amino acid sequences (Fig. 1).

There is no evidence of recombination among the brevinin sequences within the mature peptide region (*maxchi* test, $P > 0.1$), so a phylogeny-based test for positive selection is appropriate. We used PAUP* to construct a maximum likelihood phylogeny of the brevinin-1 haplotypes (Fig. 2). A phylogeny constructed using just the mature peptide region is consistent with this phylogeny (not shown). We tested for positive selection in the mature peptide region with PAML. Along the branches of this phylogeny, d_N/d_S averages 2.6. A model that employs this estimated value of d_N/d_S is significantly better than a model which forces d_N/d_S to equal one ($P < 0.05$). This result demonstrates that positive natural selection has caused much of the evolutionary diversification of these peptides.

We compared overall divergence in the intron with synonymous divergence in the mature peptide region along the branches of the tree (Fig. 2). We only obtained a small portion of the intron in the two *R. sphenocephala* sequences, so these were excluded from this analysis. Although synonymous divergence in the mature peptide region (mean $d_S = 0.012$) is more than twice as high as overall divergence in the introns (mean $d = 0.005$), the difference

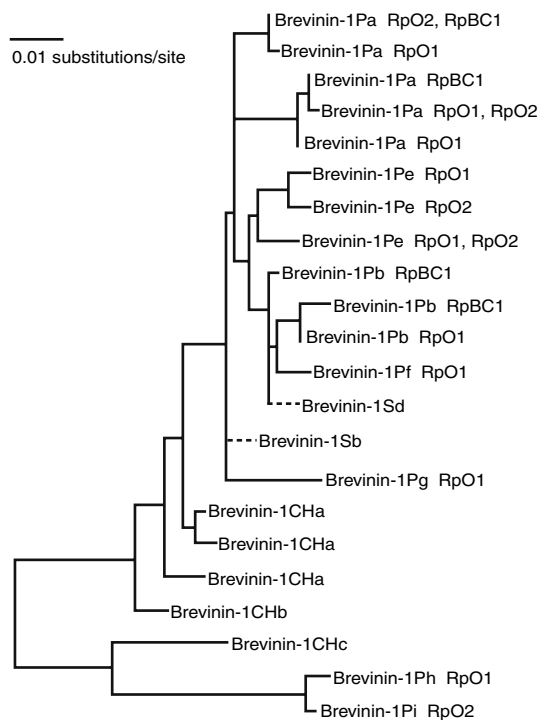


Fig. 2 Maximum-likelihood phylogram of 22 brevinin-1 haplotypes obtained from *R. pipiens*, *R. chiricahuensis*, and *R. sphenoccephala*, rooted by the midpoint. Haplotypes include part of an intron and an exon containing part of the propiece and the mature peptide region. Haplotypes are labeled according to the peptide that they encode. For *R. pipiens* haplotypes, the individual frog(s) from which the sequences were obtained are indicated (RpO1, RpO2, or RpBC1). Dotted branches leading to the *R. sphenoccephala* haplotypes indicate that branch lengths are not proportional to the other branches, since only partial sequences were obtained. In the mature peptide region, d_N/d_S averages 2.6 along the branches of this phylogeny, indicating positive natural selection. Nine haplotypes obtained from a single frog (RpO1) indicate a minimum of five brevinin-1 loci

is not significant ($P > 0.1$; paired t -test). Thus, these results provide inconclusive support for an enhanced synonymous substitution rate in the mature peptide region with respect to noncoding sequence.

Ranatuering Loci

Our initial amplification of the locus encoding ranatuering-2P resulted in a PCR product of approximately 5,000 base pairs. This was partially sequenced to reveal part of an intron, followed by the C-terminal region of the propiece, immediately followed by the mature peptide region. We designed a forward primer that annealed in the intron, Ranat2PF3. The primers Ranat2PF3 and Ranat2PR1 consistently amplified 210 nucleotides of a single locus in both *R. pipiens* and *R. chiricahuensis*, which we call the *Ranatuering2* gene since it codes for the previously

described peptide ranatuering-2P in *R. pipiens* (Goraya et al. 2000) (Fig. 1). The primers Ranat2PF4 and Ranat2PR1 consistently amplified 160 to 169 nucleotides of another locus in these species, which we call the *Ranatuering2b* gene because it is distinct from the previously described cDNA sequence of ranatuering-2Pa in *R. pipiens* (AJ427748; Chen et al. 2003) (Fig. 1).

There is no evidence of recombination among the ranatuering sequences within the mature peptide region (*maxchi* test, $P > 0.1$), so a phylogeny-based test for positive selection is appropriate. We used PAUP* to construct a maximum likelihood phylogeny of the ranatuering mature peptide region (not shown), and tested for positive selection with PAML. Along the branches of this phylogeny, d_N/d_S averages 0.6. A model which employs this estimated value of d_N/d_S is not significantly better than a model which forces d_N/d_S to equal one ($P > 0.1$). Thus, the d_N/d_S ratio provides no support for the hypothesis of positive selection acting on ranatuering.

We sequenced the *Ranatuering2* locus in 24 *R. pipiens* individuals, each from a unique population, and in four *R. chiricahuensis* individuals from two populations. We sequenced the *Ranatuering2b* locus in three *R. pipiens* individuals from three populations and in the same four *R. chiricahuensis* individuals. No intraspecific variation was observed at either ranatuering locus, with the exception of a synonymous substitution found in a single individual of *R. chiricahuensis* at *Ranatuering2b*. These results provide no support for either the hypothesis of balancing selection or the hypothesis of an enhanced mutation rate.

We tested for a recent selective sweep on the *Ranatuering2* locus by employing an HKA test (Hudson et al. 1987). We arbitrarily chose two loci, coding for arcadlin (*Arcadlin*) and type 1 myosin heavy chain (*Myosin*), as putatively neutrally evolving control sequences. The assumption of neutrality is justified because there is no reported evidence of non-neutral evolution at these loci, 41% of the sequenced nucleotides in these two loci were in introns (Fig. 3), no nonsynonymous differences were observed within or between species, and Tajima's D is not significant for either locus ($P > 0.1$ for both). We sequenced these loci in 13 of the *R. pipiens* individuals and two of the *R. chiricahuensis* individuals from which we had sequenced *Ranatuering2*. At the 139 nucleotides sequenced from *Ranatuering2*, we observed no intraspecific segregating sites among 26 *R. pipiens* sequences (all encoding ranatuering-2P), and five fixed differences (four nonsynonymous and one synonymous) with respect to the *R. chiricahuensis* sequences (all encoding ranatuering-2CHa). At the 150 nucleotides sequenced from *Myosin*, we observed 7 intraspecific segregating sites in these same individuals, and a mean of 3.5 interspecific differences. Likewise, at the 265 nucleotides of *Arcadlin*, we observed

5 intraspecific segregating sites and a mean of 2.85 interspecific differences. Divergence at the *Ranatuering2* locus is significantly greater than expected, given the amount of polymorphism in *R. pipiens* (Chi-square test, $P < 0.05$), indicating a positive selective sweep (Fig. 3). The test is still significant if only one sequence per population per locus is used (Chi-square test, $P < 0.05$), which minimizes the effect of population structure (Wakeley 1999; Ironside and Filatov 2005). It is also significant if *Ranatuering2* is just compared with *Arcadlin* (Chi-square test, $P < 0.05$) or just with *Myosin* (chi-squared test, $P < 0.05$). In contrast, the ratio of polymorphism to divergence is not significantly different between *Arcadlin* and *Myosin* (chi-squared test; $P > 0.1$).

To confirm that the selective sweep occurred in the *R. pipiens* lineage, we reconstructed the sequence of the three loci *Ranatuering2*, *Arcadlin*, and *Myosin* in the common ancestor of *R. pipiens* and *R. chiricahuensis*. We amplified *Arcadlin* and *Myosin* in *R. sphenoccephala* to infer the ancestral sequence at these loci. We were unable to amplify a *Ranatuering2* ortholog in *R. sphenoccephala*, so we used published DNA and amino acid sequences from other species to reconstruct the ancestral sequence (Fig. 1). We call the ancestral peptide ranatuering-2STa, for *Stertirana*, the clade descended from the most recent common ancestor of *R. pipiens* and *R. chiricahuensis* (Hillis and Wilcox 2005). The HKA test was then performed on the ratio of polymorphism in *R. pipiens* to divergence between *R. pipiens* and the common ancestor of *R. pipiens* and *R. chiricahuensis*. The most parsimonious reconstruction suggests that four of the five fixed interspecies differences at *Ranatuering2* occurred in the *R. pipiens* lineage. The ratio of divergence to polymorphism at *Ranatuering2* is still significantly higher than expected, even if only one

sequence per population per locus is used (Chi-square test, $P < 0.05$).

Discussion

Gene Duplication

Most animal species examined have multiple related copies of antimicrobial peptide genes, and thus a pattern of recurrent duplication might be common (Schutte et al. 2002; Maxwell et al. 2003; Semple et al. 2003; Bulmer and Crozier 2004; Lynn et al. 2004; Tennessen 2005a). Our results support this trend. These duplications are likely favored by natural selection, allowing organisms to battle pathogens with a suite of distinct peptides.

There are at least two ranatuering loci in *R. chiricahuensis*, and at least three in *R. pipiens*, if we assume that the published ranatuering-2Pa sequence (Chen et al. 2003), which we did not observe, is encoded by a unique locus (Fig. 1). We observed no allelic variation at either ranatuering locus in *R. pipiens*. Furthermore, peptides with the expected molecular weight of both ranatuering-2P and ranatuering-2Pb are expressed in the skin of *R. pipiens* from several different geographic locations (L. Rollins-Smith, personal communication), suggesting that these sequence harbor low allelic variation and are not pseudogenes. If ranatuering-2Pa were an allele at one of these loci, it would be rare and unusually divergent from the common allele. Therefore, it is probably encoded by a unique locus.

There are at least five brevinin-1 loci in *R. pipiens*, and at least three in *R. chiricahuensis*. Nine unique brevinin-1 haplotypes were each observed at least four times in RpO1. None of these nine could be formed from another multiply

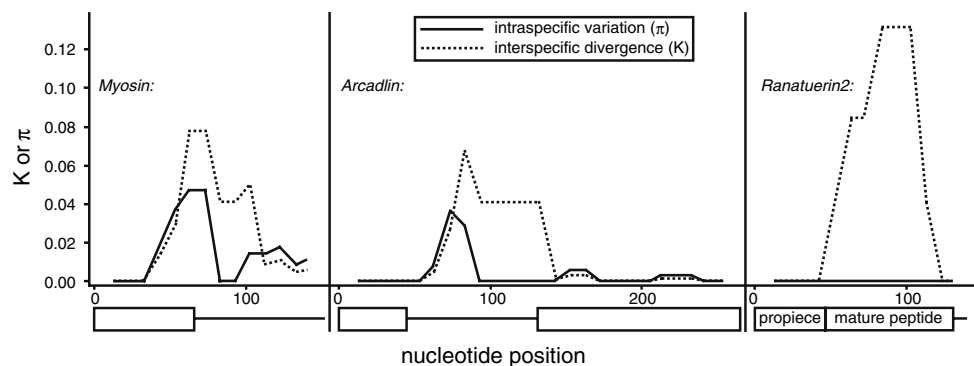


Fig. 3 Variation and divergence at three leopard frog loci: *Myosin*, *Arcadlin* and *Ranatuering2*. In *Rana pipiens*, intraspecific variation (π) is lowest at *Ranatuering2*, where no polymorphism was observed, and higher at both *Myosin* and *Arcadlin*. However, divergence between *R. pipiens* and *R. chiricahuensis* (K) is higher at *Ranatuering2* than at either *Myosin* or *Arcadlin*. Since the ratio of polymorphism to divergence should remain approximately equal throughout the

genome under neutral evolution, this suggests a recent positive selective sweep on *Ranatuering2*, if *Myosin* and *Arcadlin* are assumed to be evolving neutrally. The ratio of polymorphism to divergence is not significantly different between *Myosin* and *Arcadlin*. Window length = 25; step size = 10. White boxes beneath the graph indicate exons, and lines indicate noncoding sequence

observed haplotype with a single point mutation. Furthermore, none of them could be formed by a single recombination event between two other multiply observed haplotypes. This evidence strongly suggests that all nine are real and not PCR-induced artifacts. Therefore, since there cannot be more than two alleles at a locus, there are a minimum of five brevinin-1 loci, at least in one of the *R. pipiens* genomes. There are several possible reasons why fewer haplotypes were found in the other frogs, including homozygosity, variation in gene copy number, polymorphism at the primer binding sites, and absence by chance of certain haplotypes from the sample of clones we sequenced. Several of the brevinin-1 haplotypes in *R. pipiens* cluster together in the phylogeny, to the exclusion of the haplotypes from other species (Fig. 2). This phylogenetic pattern suggests that several duplications have occurred since *R. pipiens* speciated. Thus, leopard frog antimicrobial peptides are not merely the survivors of ancient gene duplications, but continue to be produced by diversity-generating evolutionary processes. Indeed, if intraspecific variation in gene copy number is partially responsible for the different haplotype numbers observed among individual frogs (Fig. 2), locus number might still be increasing in living populations.

The total of 22 brevinin-1 haplotypes from all frogs encodes 13 unique amino acid sequences including 12 unique putative antimicrobial peptides (Fig. 1). Three of these have previously been isolated from *R. pipiens* skin and named brevinin-1Pa, brevinin-1Pb, and brevinin-1Pe (Goraya et al. 2000), or alternatively called pipinins (Horikawa et al. 1985; Marenah et al. 2005). A fourth peptide, brevinin-1Sb, has been previously isolated from *R. sphenoccephala* skin (Conlon et al. 1999). Five of the remaining peptides, hereby named brevinin-1Pf, brevinin-1Pg, brevinin-1CHa, brevinin-1CHb, and brevinin-1Sd, closely resemble peptides which have been isolated from species in the *R. pipiens* complex (Fig. 1). The three most divergent putative peptides, brevinin-1Ph, brevinin-1Pi, and brevinin-1CHc, do not closely resemble any previously described sequence, although they are similar to each other and the genes encoding them are clearly homologous with the brevinin-1 genes. As they lack the conserved lysine-arginine motif where the mature peptide is cleaved from the propeptide (Fig. 1), it is possible that these genes do not encode antimicrobial peptides. However, the strong signature of positive selection along the branch leading to these genes suggests that they are not pseudogenes, and they might have been selected to perform another function. A six-residue deletion including one of the conserved cysteines occurs in Brevinin-1Pi, but the “Rana box” is re-established with a nonhomologous cysteine that had previously evolved (Fig. 1). We did not observe genes coding for the peptides brevinin-1Pc and brevinin-1Pd, which have

been detected in *R. pipiens* skin (Fig. 1) (Horikawa et al. 1985; Goraya et al. 2000). These might represent allelic variants or loci that do not amplify with our primers.

Leopard frogs also produce antimicrobial peptides from the temporin and esculentin families, which were not examined in this study (Goraya et al. 2000). Although the mature peptides are quite divergent, conserved gene regions demonstrate that brevinins, ranatuerins, esculentins, and temporins are homologous and have all descended from a single sequence in the ancestral ranid frog (Duda et al. 2002; Vanhoye et al. 2003). Our results demonstrate that multiple gene duplications have produced at least ten loci descended from this ancestral antimicrobial peptide gene.

Positive Selection

Antimicrobial peptide genes are thought to evolve via positive natural selection in many taxa, including frogs (Duda et al. 2002; Tennessen 2005a). Our results confirm this pattern. Evolution of the brevinin-1 family of genes is characterized by an excess of nonsynonymous substitutions in the mature peptide region, even when averaged over all codons and all branches. Although this is not true for the ranatuerin genes observed, we can infer a recent positive selective sweep on the *Ranatuerin2* locus (Fig. 3). Novel variants are probably favored by natural selection because they are more active against newly encountered pathogens or pathogens that have evolved resistance to the old antimicrobial peptide variant (Perron et al. 2006). Although we have not conclusively shown that the *in vivo* function of all the genes we observed is to encode peptides that kill pathogens, the hypothesis of an arms race with microbes would explain the high rate of adaptive evolution (Peschel and Sahl 2006).

A positive selective sweep has acted on the *Ranatuerin2* locus. No variation at this locus was observed in *R. pipiens*, but divergence is high between species, and even between *R. pipiens* and the inferred extinct sequence from the common ancestor of *R. pipiens* and *R. chiricahuensis*. It is unclear whether population structure can bias the HKA test, but we minimized any effects by sampling a single allele per locus in each population (Wakeley 1999; Ironside and Filatov 2005). Thus, positive selection has enhanced substitution in the *R. pipiens* lineage, and fixed the ranatuerin-2P peptide in that species. Interestingly, ranatuerin-2P can kill the fungus *Candida albicans* and the Gram-positive bacterium *Staphylococcus aureus* (Goraya et al. 2000), whereas the tested peptides most similar in sequence to ranatuerin-2P cannot kill these microbes (e.g., ranatuerin-2PLb and -PLd from *R. palustris*, Basir et al. 2000; ranatuerin-2ARa from *R. areolata*, Ali et al. 2002;

ranatuerin-2BYb from *R. boylii*, Conlon et al. 2003; Fig. 1). Although these human pathogens are not thought to infect frogs, it is possible that similar fungi or gram-positive bacteria were responsible for the observed selective sweep. The most likely target of the selective sweep is the histidine residue of ranatuerin-2P, where the inferred ancestral peptide had a glutamine residue (Fig. 1), since charge-changing substitutions are known to be important in antimicrobial peptide evolution (Tennessen 2005a). Selective sweeps like this one allow species with low gene flow, such as leopard frogs, to evolve collectively and maintain a cohesive species identity, even if migration is not sufficient to prevent divergence at neutral loci (Morjan and Rieseberg 2004).

Although there are several ranatuerin loci, the selective sweep at *Ranatuerin2* does not appear to have occurred immediately after gene duplication. Whereas a duplication event could have preserved ranatuerin-2STa at one locus, while allowing the other locus to evolve into ranatuerin-2P, it appears that ranatuerin-2STa has been lost. Without a sequenced genome, we cannot say conclusively that there is no gene encoding ranatuerin-2STa, but if that gene existed we would probably have amplified it with our primers. Thus, antimicrobial peptide evolution is not simply a model of recurrent duplication followed by positive selection. Rather, genes sometimes evolve adaptively without duplicating.

Allelic Variation

Intraspecific variation at antimicrobial peptide loci is not well understood, having been examined only in humans and *Drosophila* (Tennessen 2005a). We hypothesized that balancing selection might maintain multiple alleles at a locus. However, our HKA test suggests that the *Ranatuerin2* locus exhibits even less variation than expected, not more, and therefore positive but not balancing selection is acting (Fig. 3). In combination with similar results from *Drosophila* (Clark and Wang 1997), our study suggests that positive selective sweeps of new antimicrobial peptides might be common, in contrast with the highly polymorphic immunity genes such as MHC (Garrigan and Hedrick 2003; Piertney and Oliver 2006) and R-genes (Bergelson et al. 2001; Bakker et al. 2006). This trend is consistent with the hypothesis that balancing selection is more common at specialist than at generalist immunity loci (Tiffin et al. 2004). Since antimicrobial peptides interact with the microbial cell membrane instead of specific microbial gene products, it might be more difficult for microbes to evolve resistance to an effective antimicrobial peptide variant than to alleles of MHC or R-genes (Schroder 1999). Thus, an antimicrobial peptide allele can sweep to fixation before the pathogens it kills evolve resistance to it and it loses its

fitness advantage. Of course, it must lose its fitness advantage eventually, or antimicrobial peptides would not continue to be under positive selection. At this point, the old allelic variant would possibly convey higher fitness, but it cannot increase in frequency again since it has been lost. Selection will then favor new derived alleles. Thus, because selection precludes saving all potentially useful sequence variants, either as allelic polymorphism or as duplicated loci, adaptive sequence divergence is enhanced.

Different combinations of brevinin-1 haplotypes were found in three *R. pipiens* individuals (Fig. 2). Some of the haplotypes we observed are likely to be allelic variants. In addition, there appears to be polymorphism in either gene copy number or at the primer binding sites, since there are at least five loci in RpO1 but only four haplotypes were found in RpBC1. The different suites of peptides among the three frogs are quite notable. For example, brevinin-1Pg from RpO1 resembles brevinin-1Sc from *R. sphenocéphala*, but is too divergent to be allelic with any peptide found in either RpO2 or RpBC1, unless extremely strong balancing selection or introgression is the cause (Fig. 1; Fig. 2). Similarly, RpBC1 does not have any haplotype closely related to brevinin-1Ph or brevinin-1Pi (Fig. 1; Fig. 2). Although we sequenced more brevinin-1 clones from RpO1 than from RpO2 or RpBC1, this is unlikely to be the complete explanation for why we observed more haplotypes in RpO1, for the following reason. We excluded, as possible PCR artifacts, all haplotypes observed only once, as well as the only haplotype observed exactly twice (in RpO2). All of the excluded haplotypes in RpO2 and RpBC1 could be formed from a single point mutation and/or recombination between two multiply observed haplotypes in that individual. None of these haplotypes resembled the divergent haplotypes found in the other frog(s); for example, none of the excluded haplotypes in RpBC1 were anything like brevinin-1Pg, brevinin-1Ph, or brevinin-1Pi. Thus, even if we sequenced 110 clones each from RpO2 and RpBC1, we would be unlikely to observe our minimum criterion of 3 copies of anything seeming to be allelic with these missing haplotypes. Therefore, the variation in brevinin-1 haplotype numbers appears to be partly due to variation in gene copy number and/or polymorphism at the primer binding sites, at least between the Ontario and British Columbia populations. Mitochondrial DNA is distinct between *R. pipiens* from eastern and western North America, so geographic differences in brevinin-1 haplotypes are not unexpected (Hoffman and Blouin 2004).

It is unclear which of the nonsynonymous differences among brevinin-1 haplotypes represent allelic variants. Geographically distinct populations of *R. pipiens* express different combinations of brevinin-1 peptides, which could be due to nonsynonymous allelic variation (L. Rollins-Smith, personal communication). Our data suggest that

brevinin-1Ph and brevinin-1Pi are especially likely to be allelic variants (Fig. 1, Fig. 2). If these two peptides and brevinin-1CHc are encoded by the same locus, then brevinin-1Pi is a derived allelic variant of brevinin-1Ph, at a high enough frequency that frog RpO2 was homozygous for it. It remains to be seen if the six-residue difference between brevinin-1Ph and brevinin-1Pi affects the fitness of frogs. Despite the strong signature of positive selection between loci, our data do not permit us to reject the hypothesis that all polymorphism within loci is neutral. If individual antimicrobial peptide loci do not harbor much adaptive allelic variation, the alternate evolutionary strategy of frequent gene duplication appears to meet the need for a diversity of peptide variants.

Synonymous Substitution

Synonymous substitution in the mature peptide region is known to be enhanced relative to the signal sequence in frogs (Vanhoye et al. 2003; Tennessen 2005b). Enhanced synonymous divergence could be due to an increased mutation rate in the mature peptide region of the gene, or it could be that natural selection acts on synonymous sites, perhaps because of their effects on translation accuracy or mRNA folding. Our results do not allow us to evaluate these hypotheses explicitly, but it is interesting that no allelic variation was observed at *Ranantuerin2* (Fig. 3). Thus, if the mutation rate is indeed abnormally high, the selective sweep would have to have been quite recent, such that new mutations have not had time to accumulate. Alternatively, a normal mutation rate and a less recent selective sweep, coupled with selection on “silent” sites, would explain all observations.

Synonymous substitution might also be enhanced with respect to intron substitution, as is the case with flatfishes (Tennessen 2005b). The presence of an intron splitting the propiece is conserved among brevinins, ranatuerins, and the gaegurin-4 gene in *R. rugosa* (Kwon et al. 2000). Among the brevinin sequences examined, synonymous divergence in the mature peptide region is more than twice as high as overall divergence in the intron, but the difference is not significant. Thus, this study provides suggestive, but inconclusive, evidence that synonymous sites in the mature peptide region of frogs evolve faster than noncoding sites.

Conclusions

We have presented novel antimicrobial peptide gene sequences from leopard frogs, and shown that their evolution is characterized by frequent duplication and

positive selection, which reduces allelic diversity. We found no evidence of balancing selection. Our data support the trend of enhanced synonymous divergence in the mature peptide region. Thus, leopard frogs adapt to pathogens by endlessly revising their cocktail of antimicrobial peptides, resulting in a diverse but never perfect set of molecules within and among frogs. These evolutionary patterns imply that the peptides encoded by the genes discovered here might have unique functional properties, and therefore their synthesis and study would be useful.

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