Roles of Diversifying Selection and Coordinated Evolution in the Evolution of Amphibian Antimicrobial Peptides

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Antimicrobial peptides are expressed in the skin of amphibians and are used to prevent infection by microorganisms. Frog species store distinct collections of antimicrobial peptides that show variation in size, charge, conformation, and bactericidal activity, and so the evolution of antimicrobial peptide gene families may reflect the adaptive diversification of these loci. We examined the molecular evolution of antimicrobial peptide transcripts from hylid and ranid frog species. Our results show that after the gene family arose in the common ancestor of the Hylidae and Ranidae, before the divergence of these families in the Mesozoic, it subsequently diversified within these groups with numerous duplication events and divergence of loci. Moreover, we provide evidence that suggests that members of the antimicrobial peptide gene family have been subject to diversifying selection within both propiece and mature domains of hylids and solely within the mature domain of ranids. Finally, our results suggest that coordinated and compensatory amino acid replacements have occurred within the acidic propiece and cationic mature domain of hylid antimicrobial peptide precursors, as has been observed for mammalian defensin genes, but not among those of ranid precursors.

Introduction

Vertebrate immune system genes exhibit exceptionally high levels of polymorphism that is driven by selective pressure to detect a diversity of quickly evolving pathogens (Ota, Sitnikova, and Nei 2000). Excess of nonsynonymous substitutions among loci of gene families encoding the major histocompatibility complex (MHC) and immunoglobulin genes exemplify cases of diversifying selection favoring elevated amino acid sequence diversity (Hughes 1997; Hughes and Yeager 1998).

Innate (nonadaptative) immunity uses gene-encoded antimicrobial peptides to form a first line of host defense against noxious microorganisms (Nicolas and Mor 1995; Boman 1995, 1998; Andreu and Rivas 1998). Most antimicrobial peptides, either inducible or constitutive, are lethal against a broad array of microorganisms, by permeating and disrupting the membrane of target cells (Andreu and Rivas 1998; Shai 1999). Different mammalian species are equipped with sets of antimicrobial peptides ("defensins") that appear to have diversified in a species-specific manner as a result of recent gene duplication followed by evolutionary divergence (Hughes and Yeager 1997).

Dermatous glands of amphibians synthesize and store wide-spectrum antimicrobial peptides, 10–50 residues in length, that are released onto the outer layer of the skin to provide an effective and fast-acting defense against harmful microorganisms (Nicolas and Mor 1995; Simmaco, Mignogna, and Barra 1998; Amiche et al. 1999). A considerable degree of peptide polymorphism is associated with antimicrobial activity in amphibian hosts. Frogs belonging to different species or even subspecies store distinct repertoires of between 6 and 20 antimicrobial peptides, with a differing size, charge, hydrophobicity, conformation, and spectrum of action, and no two species have yet been found with the same panoply of peptide antibiotics. These patterns suggest that the amphibian antimicrobial peptide genes may be members of gene families that have been subject to diversifying selection similar to that of other immune-related genes.

On the basis of broad structural characteristics, amphibian antimicrobial peptides have been grouped into superfamilies, each being differentiated into various families. Most antimicrobial peptides from amphibians of the genus *Rana* (family Ranidae; subfamily Raninae) share a conserved disulfide bridged heptapeptide segment at the C-terminal end. Peptide families with this motif include gaegurins (24–37 residues), brevinins-1 (17–24 residues) and -2 (30–34 residues), ranalexin (20 residues), ranatuerins-1 (25 residues) and -2 (33 residues), esculentins-1 (46 residues) and -2 (37 residues), and rugosins (33–37 residues) isolated from *Rana* species from Europe, Asia, and North America (Morikawa, Hagiwara, and Nakajima 1992; Clark et al. 1994; Park, Jung, and Lee 1994; Simmaco et al. 1994; Park et al. 1995; Suzuki et al. 1995; Goraya, Knoop, and Conlon 1998). Another peptide superfamily of very short peptides composed of 10–13 residues called temporins, which do not contain the C-terminal ring, have also been characterized from European and North American *Rana* (Simmaco et al. 1996).

South American and Neotropical frogs of the Phyllomedusinae subfamily (family Hylidae) produce a rich array of linear antimicrobial peptides that adopt an amphipathic α-helical structure. They include dermaseptins B and S (24–34 residues), phylloxin (19 residues), and

dermatoxin (32 residues) from the genus *Phyllomedusa* (Amiche et al. 1994; Mor and Nicolas 1994; Charpentier et al. 1998; Amiche et al. 2000; Pierre et al. 2000) and peptides of 24–33 residues called dermaseptin-related peptides AA and PD from *Agalychnis* and *Pachymedusa* (Wechselberger 1998).

For most of the peptides described above, the cDNA-encoding precursors are known to code for a single copy of the mature antimicrobial peptide at the C-terminus of the precursor sequence. A comparison of peptide precursor sequences reveals that they have a common N-terminal preproregion, which is highly conserved both intra- and interspecifically, followed by a markedly different C-terminal domain that corresponds to the mature antimicrobial peptides (Amiche et al. 1999; Nicolas and Amiche 1999). The conserved preproregion comprises a hydrophobic signal peptide of 22 residues followed by a 16–25 residue acidic propiece which terminates by a typical prohormone processing signal Lys-Arg. The remarkable similarity of preproregions of precursors that give rise to very different antimicrobial peptides in distantly related frog species suggests that the corresponding genes form a multigene family originating from a common ancestor. The diversification of antimicrobial peptide loci could thus be part of an optimum evolutionary strategy developed by these frog species as a result of shifts to novel ecological niches when microbial predators change very rapidly.

Mammalian defensins are similar to amphibian antimicrobial peptides in that they lyse bacterial cells and are translated with signal-propiece-mature domains (Hughes and Yeager 1997). It was postulated that the cytotoxicity of mature defensins is caused by the positive net charges of these peptides (although other factors also likely play a role) and that the anionic properties of the propiece neutralize the cytotoxicity of the defensin before use (Michaelson et al. 1992). Hughes and Yeager (1997) showed that nonsynonymous substitutions that affect the net charge of the mature defensin are often associated with coordinated substitutions that affect the net charge of the propiece; for example, substitutions in the mature domain that cause an increase in the net charge of the defensin are compensated by substitutions that cause a decrease in the net charge of the propiece. Do antimicrobial peptides from amphibians show similar patterns?

In this paper we analyzed the molecular evolution of antimicrobial peptide gene families of hylid and ranid frogs. We specifically tested the hypothesis that the three domains of antimicrobial peptide transcript sequences evolve neutrally by comparing proportions of synonymous and nonsynonymous substitutions per respective site among potential orthologous or recently duplicated loci. We also assessed whether coordinated amino acid changes characterize the evolution of amphibian antimicrobial peptides by examining patterns of charge-altering nonsynonymous substitutions among sequences and ancestral sequence predictions.

Methods

Nucleotide sequences of 18 dermaseptins, dermaseptin-related peptides, dermatoxins, and phylloxins from the hylids *Agalychnis annae* (GenBank accession numbers AJ005183–AJ005188), *Pachymedusa dacnicolor* (AJ005189–AJ005193), and *Phyllomedusa bicolor* (AJ251875, AJ251876, X72387, X70278, and Y16564–Y16566) were obtained from GenBank. Nucleotide sequences of 11 brevinins, esculentins, gaegurins, ranalexins, and temporins from the ranids *Rana catesbeiana* (GenBank accession number S69903) *R. esculenta* (X77831–X77833), *R. rugosa* (U22392 and U22393), and *R. temporaria* (AJ251566, AJ251567, and Y09393–Y09395) were also obtained from GenBank.

We aligned the nucleotide sequences of the antimicrobial peptide transcripts from the two frog families with ClustalX (Thompson et al. 1997) and by eye. First, we aligned the predicted amino acid sequences of the different domains of the peptides and the nucleotide sequences of the 5′ and 3′ untranslated regions (UTR) separately with ClustalX. Then, the nucleotide sequences of the different regions were joined, and final adjustments to the alignment were made manually. The alignments are available as *Supplementary Material.*

We used Modeltest 3.0 (Posada and Crandall 1998) to determine the models of nucleotide substitutions that best fit our data sets for phylogenetic reconstruction. Molecular phylograms from the alignments were determined with Neighbor-Joining using PAUP* (Swofford 1999). Levels of support for branches were estimated with bootstrapping methods (1,000 replicates) also with PAUP* (Swofford 1999). To interpret the origins of these gene families, we examined the topologies of the phylogram; we assume that the sequences represent distinct loci in the species sampled.

To determine if diversifying selection operates among members of antimicrobial gene families in frog species, we estimated the proportions of nonsynonymous substitutions (d_N) and synonymous substitutions (d_S) per respective site among sequences with maximum-likelihood (ML) methods (Yang 1998) among potential recently duplicated or orthologous loci. These values were calculated among terminal and predicted ancestral node sequences for each peptide domain using PAML (Yang 1997). Ancestral nodes were also predicted using parsimony and ML methods with PAUP*; these predictions were compared with those determined with PAML. We performed likelihood ratio tests to determine if ratios of d_N to d_S (ω) exceed a value of 1 by comparing twice the difference between the log-likelihoods of the null model (ω is fixed at 1) and the alternative model (ω is a free parameter) to a χ^2 distribution with one degree of freedom (Yang and Bielawski 2000). Many of the mature antimicrobial peptides are known to be or suspected of being carboxamidated at the C-terminus during processing; this results in the exclusion of two or three of the terminal amino acid residues of ranid and hylid sequences, respectively. Therefore, the final two or three codons of the mature domain of ranid and hylid sequences, respectively, were excluded from these analyses.

We estimated the proportions of radical (p_{NR}) and conservative (p_{NC}) nonsynonymous substitutions with respect to net charge among terminal and ancestral pre-

dicted node sequences among the same sets of sequences as analyzed for d_S and d_N within propiece and mature domains, according to the methods of Hughes, Ota, and Nei (1990). We omitted the final two or three codons from these analyses when they were suspected of being excluded during processing. We tested whether p_{NR} was significantly greater than p_{NC} by conducting a one-tailed t-test with infinite degrees of freedom.

Net charges of propiece and mature peptides were compared to determine if the charges of these domains showed a negative relationship. Net charges were calculated based on numbers of positively (arginine, histidine, and lysine) and negatively charged residues (aspartic and glutamic acid) in the peptides. As with estimates of d_N, d_S, p_{NR}, and p_{NC}, we calculated the net charges of mature domains while excluding codons corresponding to the two or three terminal residues of ranid and hylid sequences, respectively.

Results and Discussion

Our results show that the genes encoding antimicrobial peptides of hylid and ranid frogs are members of a large gene family whose history has been characterized by numerous duplication events and the subsequent evolutionary divergence of these loci. Results from comparisons of the proportions of nonsynonymous and synonymous substitutions among antimicrobial peptide transcript sequences suggest that diversifying selection operates among these loci as it does among other immunodefense-related genes (Hughes 1997; Hughes and Yeager 1997, 1998; Ota, Sitnikova and Nei 2000), particularly in the mature domain. Moreover, coordinated amino acid changes appear to have occurred within propiece and mature domains of antimicrobial peptide genes in hylids but not in ranids, suggesting different roles of propiece peptides in these families.

Gene Family History

The Hasegawa, Kishino, and Yano (1985) (HKY) model of nucleotide substitutions with gamma correction was the model of sequence evolution that best fit both the hylid and ranid data sets (fig. 1). The phylogram of hylid sequences is not completely resolved, but several distinct clades are apparent. Sequences from *A. annae* and *P. dacnicolor* cluster tightly, together with strong bootstrap support in three cases, DRP AA-1-1–DRP PD-1-5, DRP AA-2-5–DRP PD-3-6, and DRP AA-3-6–DRP PD-3-3 (fig. 1), suggesting that these sequences may represent orthologous loci in these species.

Within the ranid phylogram, there are two distinct clades of sequences: a clade consisting of a brevinin from *R. esculenta*, a gaegurin from *R. rugosa*, ranalexin from *R. catesbeiana*, and temporins from *R. temporaria* supported by a bootstrap value of 98%; and a clade consisting of brevinins from *R. esculenta* and *R. temporaria* and a gaegurin from *R. rugosa* supported by a bootstrap value of 96% (fig. 1). Relationships of the three brevinins in the first clade and of the temporins in the second suggest that brevinin 2Ta and 2Tb and temporin B and
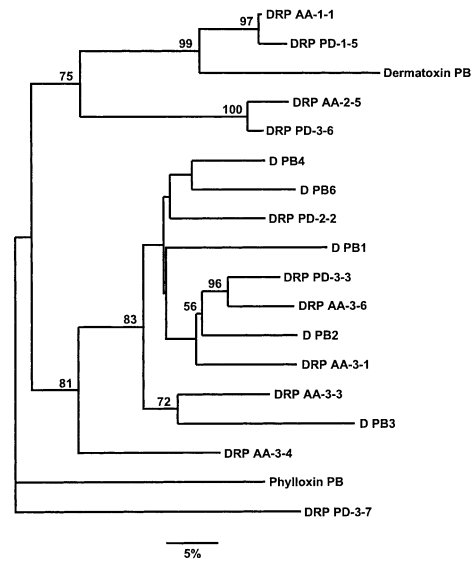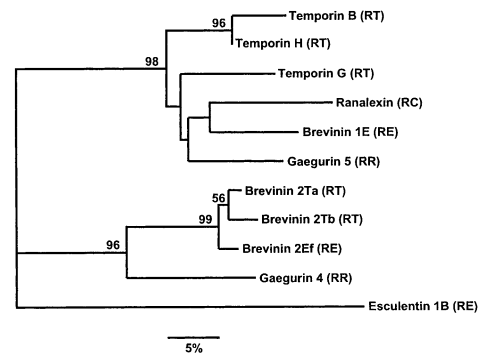

FIG. 1.—Molecular phylograms of nucleotide sequences of hylid and ranid antimicrobial peptide transcripts as reconstructed with neighbor-joining methods of HKY distances. The HKY model with gamma correction was used for the reconstruction of each data set. Parameters used for the hylid phylogram reconstruction are transition to transversion ratio (Ti/Tv) = 1.0011, proportion of invariable sites (I) = 0, and the shape parameter of the gamma distribution (g) = 0.8838. Parameters used for the hylid phylogram reconstruction are Ti/Tv = 1.1980, I = 0, and g = 0.6694. Phylograms are midpoint rooted. Bootstrap values from 1,000 replicates greater than 50% are indicated on branches. Among the hylid sequences, D = dermaseptin; DRP = dermaseptin-related peptide; sequence names were appended with AA, PD, and PB to show that the sequences were identified from *A. annae*, *P. dacnicolor*, and *P. bicolor*, respectively. Among the ranid sequences, the brevinins are from *R. temporaria* (RT) (2Ta and 2Tb) and *R. esculenta* (RE) (1Ef and 2Ef); esculentin 1B is from *R. esculenta*, gaegurins 4 and 5 are from *R. rugosa* (RR); ranalexin is from *R. catesbeiana* (RC); and the temporins are from *R. temporaria* (RT); species from which the sequences were identified are indicated with abbreviated sequence names in parentheses.

H in *R. temporaria* are recently duplicated loci in this species.

Hylid and ranid families diverged during the Mesozoic, though precise dating of this event is controversial (see Duellman and Trueb 1994, pp. 472–495; Feller and Hedges 1998), giving rise to very distinct evolutionary histories and geographical distributions. The diversity of modern antimicrobial peptide loci present in these families reflects the origination and divergence of

Table 1
Maximum-likelihood Estimates of Synonymous (d_S) and Nonsynonymous (d_N) Substitutions Per Respective Site and Estimated Proportions of Conservative (p_{NC}) and Radical (p_{NR}) Nonsynonymous Substitutions (with respect to charge) Among Terminal and Predicted Node Sequences (see fig. 1) Within Signal, Propiece, and Mature Domains.

COMPARISON TO NODE SEQUENCE	SIGNAL				PROPIECE				MATURE			
	d_S	d_N	p_{NC}	p_{NR}	d_S	d_N	p_{NC}	p_{NR}	d_S	d_N	p_{NC}	p_{NR}
Hylid sequences												
DRP AA-1-1 ..	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
DRP PD-1-5 ..	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.2	0.0	0.0	0.0
DRP AA-2-5 ..	**0.2**	**2.3**	2.6	0.0	0.0	0.0	0.0	0.0	10.6	4.7	3.0	0.0
DRP PD-3-6 ..	4.3	0.0	0.0	0.0	**0.0**	**3.9**	**0.0**	**4.5****	**0.3**	7.5*	**6.0**	**14.5***
Ranid sequences												
Brevinin 2Ta ..	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.8	5.5	5.3	3.4
Brevinin 2Tb ..	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**0.3**	**6.6*****	**4.3**	**8.8**
Brevinin 2Ef ..	**0.0**	**2.2**	2.4	0.0	10.0	2.2	6.3	0.0	**0.1**	**4.5*****	4.5	3.4
Temporin B ...	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Temporin H ...	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**0.1**	**5.6*****	6.3	0.0

NOTE.—Values are presented as percentages. Cases where d_N or p_{NR} is greater than d_S or p_{NC}, respectively, are indicated with values in bold. Asterisks show cases in which the d_N to d_S ratio is significantly greater than 1, or p_{NR} is significantly greater than p_{NC} (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$)

these genes since the Mesozoic. As shown in the phylograms, sequences do not cluster according to species; for example, sequences of *A. annae* occur throughout the hylid phylogram (fig. 1). This pattern implies that many antimicrobial peptide loci originated before the divergence of the species sampled and that concerted evolution has played little role in the evolution of this gene family. Only in a few cases do loci appear to be the result of recent duplications; that is, only in two cases do sequences from the same species uniquely cluster together, brevinin 2Ta and 2Tb and temporin B and H from *R. temporaria* (fig. 1).

Adaptive Evolution

We examined patterns of nucleotide substitutions within each of the three peptide domains among four sets of sequences that may represent orthologous or recently duplicated loci: DRP AA-1-1 and PD-1-5 (pairwise HKY distance = 0.031) and DRP AA-2-5 and PD-3-6 (HKY distance = 0.056) from the hylids *A. annae* and *P. dacnicolor*; brevinins 2Ta, 2Tb, and 2Ef from the ranids *R. esculenta* and *R. temporaria* (HKY distances range between 0.041 and 0.052); and temporins B and H from *R. temporaria* (HKY distance = 0.013). We did not include DRP AA-3-6 and PD-3-3 in these analyses because the level of divergence among these sequences was more than two times greater (HKY distance = 0.116) than those observed among the other pairs from these species, suggesting that DRP AA-3-6 and PD-3-3 are not orthologous.

We estimated d_S and d_N along branches among terminal and predicted ancestral node sequences for each domain with PAML (Yang 1997). Because our phylograms were not completely resolved, we did not estimate parameters with PAML for complete data sets; d_S and d_N were calculated using three subsets of sequences as described subsequently. The first subset of sequences included sequence pairs DRP AA-1-1–PD-1-5 and DRP AA-2-5–PD-3-6 and dermatoxin PB (see fig. 1). The

second subset included brevinins 2Ta, 2Tb, 2Ef, and gaegurin 4. The final subset included the sequence pair temporin B and H plus brevinin 1E, gaegurin 5, ranalexin, and temporin G; because the topology of the last four sequences is not well supported (see fig. 1), the user-supplied tree was constructed as a polytomy with regard to these sequences. Where differences occurred between terminal and predicted ancestral node sequences with PAML, we compared the ancestral predictions with those generated by parsimony and ML methods with PAUP* (Swofford 1999), and in all cases the methods gave similar if not identical predictions of ancestral node sequences.

Results from analyses of ML estimates of d_S and d_N show that patterns of substitutions are not equivalent among the three peptide domains (table 1). Among hylid sequences, d_S and especially d_N increase from the signal to propiece to mature domain. Among the first set of sequences from hylids, DRP AA-1-1 and PD-1-5, there are no nonsynonymous substitutions within any domain among these and the predicted ancestral node sequence; the only differences are synonymous substitutions within the mature domain among DRP PD-1-5 and the predicted ancestral node sequence. The lack of nonsynonymous divergence among these sequences shows that some antimicrobial loci are under strong purifying selection.

On the contrary, among the second set of hylid sequences, DRP AA-2-5 and PD-3-6, d_N exceeds d_S and d_N to d_S ratios (ω) are greater than 1 within the propiece and mature domains among DRP PD-3-6 and the ancestral node sequence (table 1); ω is significantly greater than 1 within the mature domain. These results suggest that the DRP PD-3-6 locus has been subject to diversifying selection potentially within the propiece domain and significantly within the mature peptide domain.

All nonsynonymous substitutions within the propiece and most within the mature domain among DRP PD-3-6 and the ancestral node sequence are radical nu-

Propiece domain

DRP PD-3-6
```
GAA GCA GAA AAA AGA GAA GAG GAA AAT GAA GAG AAA CAA GAA GAC GAT GAT GAA AGT GAA AAG AAG AGA
 E   A   E   K   R   E   E   E   N   E   E   K   Q   E   D   D   D   E   S   E   K   K   R
```

Ancestral node
```
GAA GAA GAA AAA AGA GAA GAG GAA AAT GAA GAG AAA CAA GAA GAC GAT GAT CAA AGT GAA AAG AAG AGA
 E   E   E   K   R   E   E   E   N   E   E   K   Q   E   D   D   D   Q   S   E   K   K   R
```

Mature domain

DRP PD-3-6
```
GGG GTG GTG ACA GAT TTA TTA AAT ACT GCT GGA GGT CTG CTT GGA AAT CTG GTT GGT TCA CTG TCT GGT
 G   V   V   T   D   L   L   N   T   A   G   G   L   L   G   N   L   V   G   S   L   S   G
```

Ancestral node
```
GGG CTG GTG ACA GGT TTA TTA AAT ACT GCT GGA GGT CTG CTT GGA GAT CTG CTT GGT TCA CTG TCT GGT
 G   L   V   T   G   L   L   N   T   A   G   G   L   L   G   D   L   L   G   S   L   S   G
```

FIG. 2.—Nucleotide and amino acid differences among DRP PD-3-6 and the predicted ancestral node sequence. Single-letter amino abbreviations are used. All amino acid differences are underlined; amino acid differences that result in a change in net charge of the peptide are also in bold.

cleotide substitutions that affect the net charges of propiece and mature peptides (table 1). In both these cases, the proportion of radical nonsynonymous substitutions (p_{NR}) is significantly greater than that of conservative nonsynonymous substitutions (p_{NC}) (table 1). These substitutions account for two charge-altering amino acid differences within both the propiece and the mature peptides that essentially result in no change of the net charges of these peptides (fig. 2). The pattern and mode of nucleotide substitutions in the propiece and mature domains suggest that substitutions in these domains may be coordinated and compensatory among hylid antimicrobial peptides (see subsequently).

Among ranid sequences, both d_S and d_N also generally increase from the signal to propiece to mature domains; the largest values of d_N were measured exclusively within the mature domain (table 1). In three cases, d_N is greater than d_S within the mature domain, and ω is significantly greater than 1. In only one case, d_N exceeds d_S within the signal domain, but ω is not significantly greater than 1. Values of d_N do not exceed d_S in any case, and in most cases d_N equals 0 within the propiece domain for ranid sequences. These results suggest that diversifying selection has operated within the mature domain of some antimicrobial peptide loci in ranids, but propiece domains appear to be strictly under purifying selection.

Very few of the nonsynonymous substitutions are radical among the ranid and ancestral node sequences (table 1). Values of p_{NR} exceed those of p_{NC} in only one case, within the mature domain among brevinin 2Tb and the ancestral node sequence, but this difference is not significant (table 1). This evidence implies that although mature peptide domains are subject to diversifying selection within both hylids and ranids, selection plays a much different role among the antimicrobial peptide loci of these two groups, and it does not appear that propiece and mature peptides evolve in a coordinated manner within Ranidae (see subsequently).

We clearly performed multiple tests in comparing ω and values of p_{NR} and p_{NC} (table 1), and so in some cases the significant outcomes may simply be the result of chance. However, the patterns we observed, particularly the higher values of d_N in the mature domain and the lower values in the other domains among hylid and ranid ancestral sequence comparisons (table 1), suggest that amphibian antimicrobial peptides evolve adaptively.

Coordinated Evolution

The propiece and mature domains of mammalian defensins have been shown to evolve in a coordinated manner (Hughes and Yeager 1997). This phenomenon is revealed by a negative relationship among the net charges of propiece and mature peptides. If the evolution of propiece and mature domains of amphibian antimicrobial peptides is also coordinated, we expect to find a negative relationship among net charges of these peptides. Such a relationship was observed among antimicrobial peptides from hylids but not from ranids (fig. 3). Net charges of the amino acid sequences of propiece and mature antimicrobial peptides from hylids range from −11 to −4 and from −2 to +6, respectively. Among ranids, they range from −9 to −4 and from +1 to +5 (fig. 3). The net charges of propiece and mature peptides show a negative relationship among hylid sequences (slope = −0.58 and is significantly different from 0, $P = 0.014$), but they show no relationship among ranid sequences (slope = 0.09 and is not significantly different from 0, $P = 0.802$) (fig. 3). These results suggest that opposing charges of propiece and mature peptides are associated among hylid but not among ranid antimicrobial peptide loci, as might be expected from the patterns of substitutions of these loci mentioned previously.

If substitutions are coordinated and compensatory, charge-altering nucleotide substitutions will have occurred concurrently within both propiece and mature domains. On the basis of the prediction of the ancestral node sequence of hylid sequence DRP PD-3-6 from *P. dacnicolor* (as determined with PAML), we show that two charge-altering nucleotide substitutions occurred in both propiece and mature domains in the gene lineage that gave rise to this locus (fig. 2). Ancestral node predictions using parsimony and ML methods with PAUP* show identical results. These substitutions are compensatory in the sense that in both domains they account for no change in the net charges of propiece and mature peptides. Moreover, ω is greater than 1, and p_{NR} is significantly greater than p_{NC} within both these domains (table 1). It should be noted that the chronological order
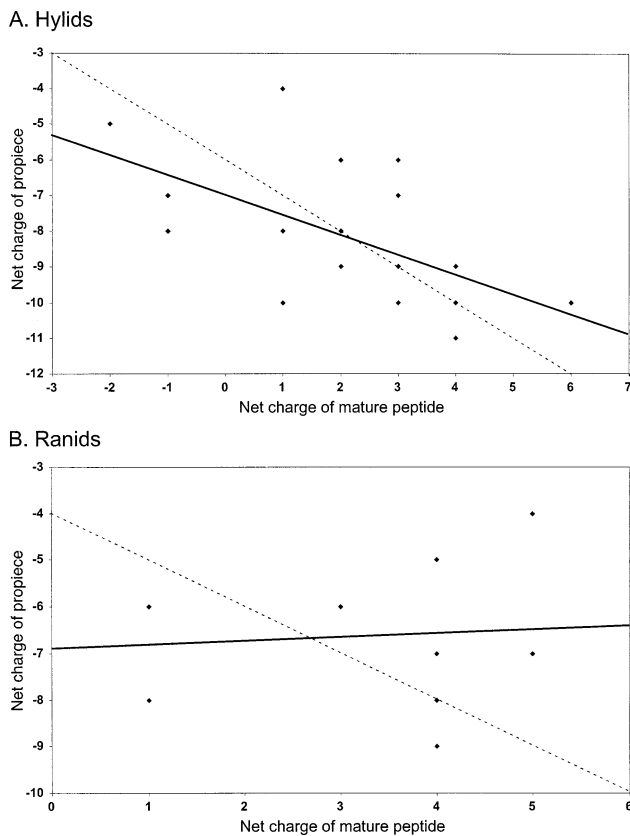
A. Hylids

B. Ranids

Fig. 3.—*A*, Net charge of the propiece is negatively related to the net charge of mature peptide for sequences from hylids (Y = −2.64 − 0.58X; the regression line is plotted as a solid line, the dashed line is plotted at a slope corresponding to 45°). *B*, Net charge of the propiece does not show any relationship to that of the mature peptide for sequences from ranids (Y = −3.89 + 0.09X; the regression line is plotted as a solid line, the dashed line is plotted at a slope corresponding to 45°).

of the charge-altering substitutions within propiece and mature domains is unknown, and so although the substitutions appear to be coordinated and compensatory, they may not be so. Future work should be directed at analyses of orthologous loci of DRP PD-3-6 in close relatives of *P. dacnicolor* to verify the occurrence of coordinated evolution at this locus.

Summary

Our results suggest that diversifying selection has operated within the mature domain of some antimicrobial peptide loci of hylid and ranid amphibians. Because the peptides that members of this gene family encode are used to protect against noxious microbes, their adaptive evolution may be caused by several factors. Indeed, results from functional assays previously conducted show that amphibian antimicrobial peptides have different bactericidal activities and are specific for different types of microorganisms (Simmaco, Mignogna, and Barra 1998). As with MHC receptors, immunoglobulins, and defensins (Hughes 1997; Hughes and Yeager 1997, 1998; Ota, Sitnikova, and Nei 2000), antimicrobial peptides may be under selection directed by the evolution of pathogens. For antimicrobial peptides this selection

may be in response to the evolution of the cellular membranes of microbes to prevent disruption by antimicrobial peptides. Alternatively, frog species may be exposed to different microorganisms in different habitats or environments such that the plethora of expressed antimicrobial peptides have evolved for the particular microbial biota that these species encounter. Such hypotheses cannot be tested without further analyses of the evolution and expression of antimicrobial peptide gene families among closely related hylid and ranid species, surveys of the communities of microbes with which these frogs are associated, and further functional assays and investigations of the activities of these peptides.

Our analyses also show that antimicrobial peptide loci have evolved differently among hylids and ranids. Within both groups, diversifying selection has operated within the mature domain; within hylids, the propiece domain potentially appears to have been subject to diversifying selection. The results also suggest that coordinated and compensatory amino acid replacements in the propiece and mature domains may have occurred among antimicrobial peptide loci from hylids but not in ranids.

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