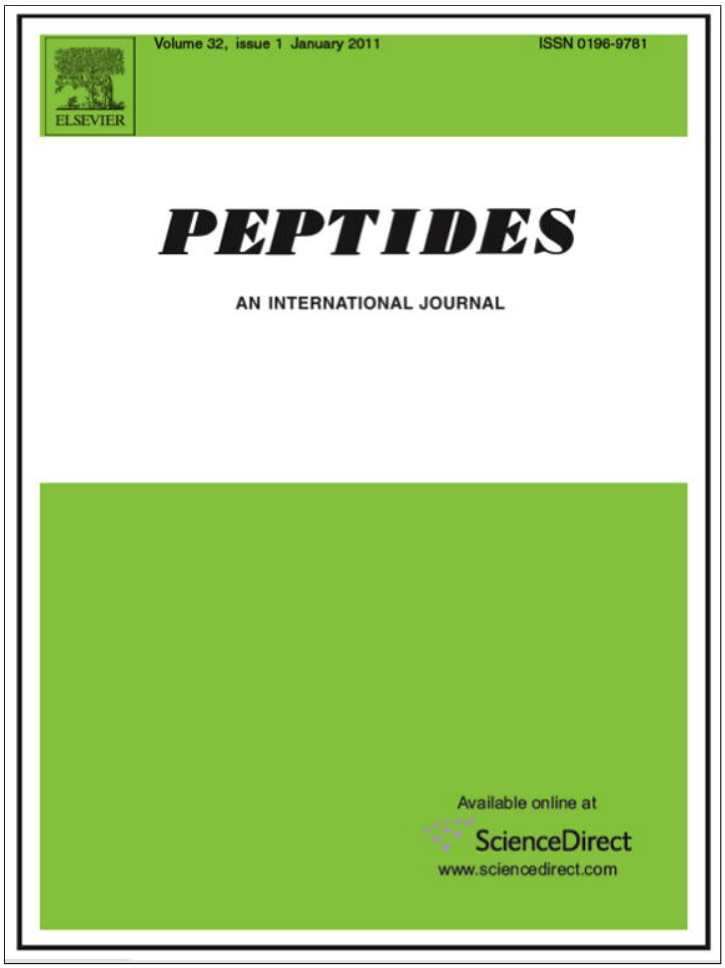

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Evidence for convergent evolution in the antimicrobial peptide system in anuran amphibians

Enrico König[a,*], Olaf R.P. Bininda-Emonds[b]

[a] Institut für Spezielle Zoologie und Evolutionsbiologie mit Phyletischem Museum, Friedrich-Schiller-Universität Jena, Erbertstrasse 1, 07743 Jena, Germany
[b] AG Systematik und Evolutionsbiologie, IBU – Fakultät V, Carl von Ossietzky Universität Oldenburg, Carl von Ossietzky Strasse 9–11, 26111 Oldenburg, Germany

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ABSTRACT

Amphibians are characterized in part by their highly specialized and glandular skin that enables key physiological functions such as cutaneous respiration and defense against a variety of micro- and macroscopic predators via toxic components (e.g., alkaloids and bufodienolids), biogenic amines, neuropeptides and antimicrobial peptides (AMPs). To date, DNA sequence information regarding AMP genes in anurans is restricted to only a few anuran families and largely to "higher frogs" (Neobatrachia). Here, we analyze the DNA information for the signal sequences of the AMP precursors in anuran amphibians available to the end of 2009 in an explicit phylogenetic framework to characterize the evolution of this large, diverse gene family. Comparison of cDNA sequences suggests that there are at least three different motifs within the signal peptide sequence of the AMP-precursor corresponding to the evolutionary lineages Neobatrachia, Bombinatoridae (*Bombina* spp.) and Pipidae (*Xenopus laevis*). The signal sequences are strongly conserved within each lineage (as previously noted for Neobatrachia), but highly divergent between them. Together with the lack of a linear relationship between the degree of sequence divergence and evolutionary time, we hypothesize that the anuran AMP system has evolved convergently on at least three occasions. However, additional sampling, especially among the largely poorly sampled non-neobatrachian lineages, is required to confirm this hypothesis and could reveal the existence of additional signal sequence motifs.

1. Introduction

Anurans (frogs and toads) possess an efficient defense system based on bioactive compounds in their skin secretions. Included among these compounds are both toxic molecules (e.g., alkaloids or bufotoxins) and biogenic amines to protect against predation from large animals, including birds, snakes and mammals [20,23]. In addition, research over the past two decades has uncovered numerous peptides in the skin secretions [3,31,38]. Although many of these peptides are of unknown function, others show neuroactive or antimicrobial activity [3,23,38] and so seem to comprise a major component of the defensive skin secretion of frogs.

In this paper, we restrict our focus to antimicrobial peptides (AMPs), which are widely distributed in the animal kingdom (e.g., horseshoe crabs, insects, mammals and humans) and are considered to be part of the innate immune system as a first line of defense against invading pathogens (e.g., gram-positive and -negative bacteria, fungi and protozoans) [5,40,47]. In anuran amphibians, AMPs have been isolated from many species, including representatives of the two major groups, Neobatrachia ("higher frogs") and the polyphyletic "Archaeobatrachia" ("archaic frogs"). Within the former group, AMPs are best characterized for the families Ranidae and Hylidae (known for 56 and 36 species, respectively), but are also known from species belonging to numerous other families (e.g., Dicroglossidae, Hyperoliidae, Myobatrachidae and Leptodactylidae). Similarly, AMPs have been documented throughout "Archaeobatrachia", including Ascaphidae, Alytidae, Bombinatoridae and Pipidae. However, several lineages from both archaic and higher frogs (e.g., Pelobatidae, Scaphiopodidae, Ceratophryidae, Microhylidae, Pyxicephalidae) appear to lack an AMP system suggesting a more sporadic distribution of host-defence peptides in Anura [11]. Moreoever, those peptides with antimicrobial activity found in the stomach tissue of *Bufo bufo gargarizans* [36] and in *Racophorus schlegelii* (Racophoridae) [32] are instead cleavage products of histone H2A and H2B, respectively. However, the latter two proteins are neither products of the glandular skin nor cationic and α-helical peptides as is the case for classic AMPs.

Because of the obvious potential of AMPs for pharmaceutical application [5,7,10,48], investigations of these defensive peptides to date have largely been restricted into the elucidation of their chemical structures and biological functions, with molecular cloning revealing a common tripartite signal-spacer-peptide

* Corresponding author. Tel.: +49 441 7983373.
E-mail address: en.koenig@uni-oldenburg.de (E. König).

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structure [44] of the entire prepropeptide (precursor). By contrast, research into the evolution of anuran AMP genes, despite an ever-increasing database of molecular data, has been rare. Much of this research in frogs has been restricted to Neobatrachia, for which the vast majority of the known sequence data is available. These studies infer a common ancestry of the neobatrachian AMP gene dating to 150 million years (Ma) ago based on the high degree of conservation within its N-terminal signal peptides across the group [44]. By contrast, the actual gene products, the mature peptides that interact with and disrupt invading pathogens, evolve adaptively via positive selection, leading to an extraordinary diversity of structurally and functionally different peptide families, even among closely related species [21,42]. Even so, Michael Conlon and colleagues showed that a comparison of the active peptides themselves does tend to reflect phylogenetic relationships between closely related species in Ranidae [19,15,18,10,17].

Analogous information about anuran AMP genes in archaeobatrachian frogs is much more sparse, being largely restricted to the genera *Bombina* and *Xenopus*, and has never been investigated in an evolutionary context. Genes in this group also classically display the stereotypical signal-spacer-peptide precursor structure and an apparently conserved signal peptide exists [3,27,30]. One notable structural difference, however, is that many archaeobatrachian AMP genes uniquely show tandem repeats of the spacer-peptide subunits [27,30,46].

As is typical of many immune system related components, there appears to be rapid evolution within the anuran AMP system, with most species being characterized by a unique cocktail of peptides. It is typically held that this evolution occurs via gene duplication, with the different paralogs forming a large gene family [21,44] and distinguished by the variable spacer and hypervariable peptide regions. The signal sequence, by contrast, is commonly viewed as showing a high degree of sequence conservation, as has been shown for neobatrachians [44], presumably due to its constraining biological functions (e.g., cell targeting and peptide processing). These two components are reflected in the genetic structure of the AMP gene, which comprises discrete exons corresponding to the conserved (signal sequence and the first three amino acids of the spacer) and variable parts (remaining spacer plus peptide domain) of the entire gene [6,24,34].

Whereas most of the research attention for the AMP system has focused on the hypervariable AMP protein itself, we instead focus exclusively on the signal sequence of the AMP gene, using DNA sequence data available to the end of 2009 to provide an evolutionary overview of the anuran AMP system. Based on the significant differences in these sequences between higher anuran taxa that we observed, we hypothesize that the evolution of AMP genes has occurred independently in at least three different lineages of anuran amphibians, thereby mirroring the convergent evolution of AMPs across the animal kingdom on a more local scale.

2. Materials and methods

Amino-acid and cDNA sequences for AMPs were obtained initially from the review of Pukala et al. [38] and supplemented by BLASTing [1] these data, both as nucleotides (blastn) and translated nucleotides (tblastx), against GenBank to discover more recently described AMP sequences. Only cDNA sequences corresponding to the entire precursor were examined. Alignment of the signal sequences was performed initially on an amino-acid level using Clustal W v2.0.10 [43] in combination with transAlign.pl [4] before manual adjustment. The aligned dataset is available from the authors on request and follows the proposed nomenclatural systems of Conlon [8,9] and Amiche et al. [2] to designate AMPs wherever possible. Otherwise original names were used as published.

Pairwise distances between all cDNA sequences were calculated using PAUP* v4.0b10 [41] according to the optimal model of evolution for the data (TVM + I + G) as determined using ModelTEST v3.7 [37]. Each pairwise distance was then related to the evolutionary time separating the two species as far as could be reconstructed from the phylogenies and divergence-time estimates in Frost et al. [26] and Roelants et al. [39]. For the latter, we assumed a divergence time of one Ma for pairwise comparisons of distinct signal sequences from the same species. Given the highly unstable nature of anuran taxonomy, our procedure here was conservative: pairwise comparisons for which divergence time estimates could not be inferred reliably were excluded from the analyses. All statistical analyses were performed using PAST v2.01 [28].

Finally, all signal sequences were BLASTed against the recently released genome sequence for *Xenopus (Silurana) tropicalis* [29] to establish the presence versus absence of each of the different hypothesized motifs in a largely complete anuran genome. To accomplish this, all 187,738 scaffolds associated with the *Silurana tropicalis* genome (AAMC01100001–AAMC01190823) were downloaded from GenBank and used to construct a local BLAST database using formatdb v2.2.19 (http://blast.ncbi.nlm.nih.gov/Blast.cgi). We then searched against this database using each of the 356 available DNA signal sequences in turn as a query sequence using blastall v2.2.19. BLAST searches were again performed on both the nucleotide (blastn) and translated nucleotide (tblastx) levels.

3. Results

3.1. Data set

The Pukala et al. [38] data set (310 AMPs from 60 species) supplemented with BLAST searches of GenBank (up to the end of 2009) resulted in a total of 840 AMPs from 113 species (summarized in Table 1) for which 383 cDNA sequences from 47 species were available. Of these 47 species, 43 are neobatrachians (91%), with the remaining four archaeobatrachian species (*Xenopus laevis*, *Bombina maxima*, *Bombina orientalis*, and *Bombina variegata*) contributing 63 of the 383 precursor sequences (16%) to the data set.

3.2. Structural characteristics of signal sequences (Fig. 1)

All signal peptides are predominantly hydrophobic and, except for the Maximin-S precursors from *Bombina maxima* [45] and possibly the incompletely sequenced Phylloseptin-H2 from *Phyllomedusa hypochondrialis*, contain at least one site with a positively charged lysine residue near their N-terminus. Generally, a minimum of two polar amino acids can be found in all signal peptides, with acidic residues being absent. Two exceptions here are Dermatoxin-A1 and Dermatoxin-DA1 from *Agalychnis annae*

Table 1
Number of AMP sequences (as amino acids) and corresponding precursors (as cDNA) among anuran families known as of the end of 2009.

Family	AMPs		cDNA	
	Sequences	Species	Sequences	Species
Leiopelmatidae	14	2	–	–
Pipidae	12	2	5	1
Alytidae	7	1	–	–
Bombinatoridae	52	3	58	3
Myobatrachidae	19	4	–	–
Hylidae	216	36	73	11
Leptodactylidae	14	6	–	–
Hyperoliidae	6	2	5	2
Dicroglossidae	10	3	6	2
Ranidae	503	56	236	28
Total	853	115	383	47

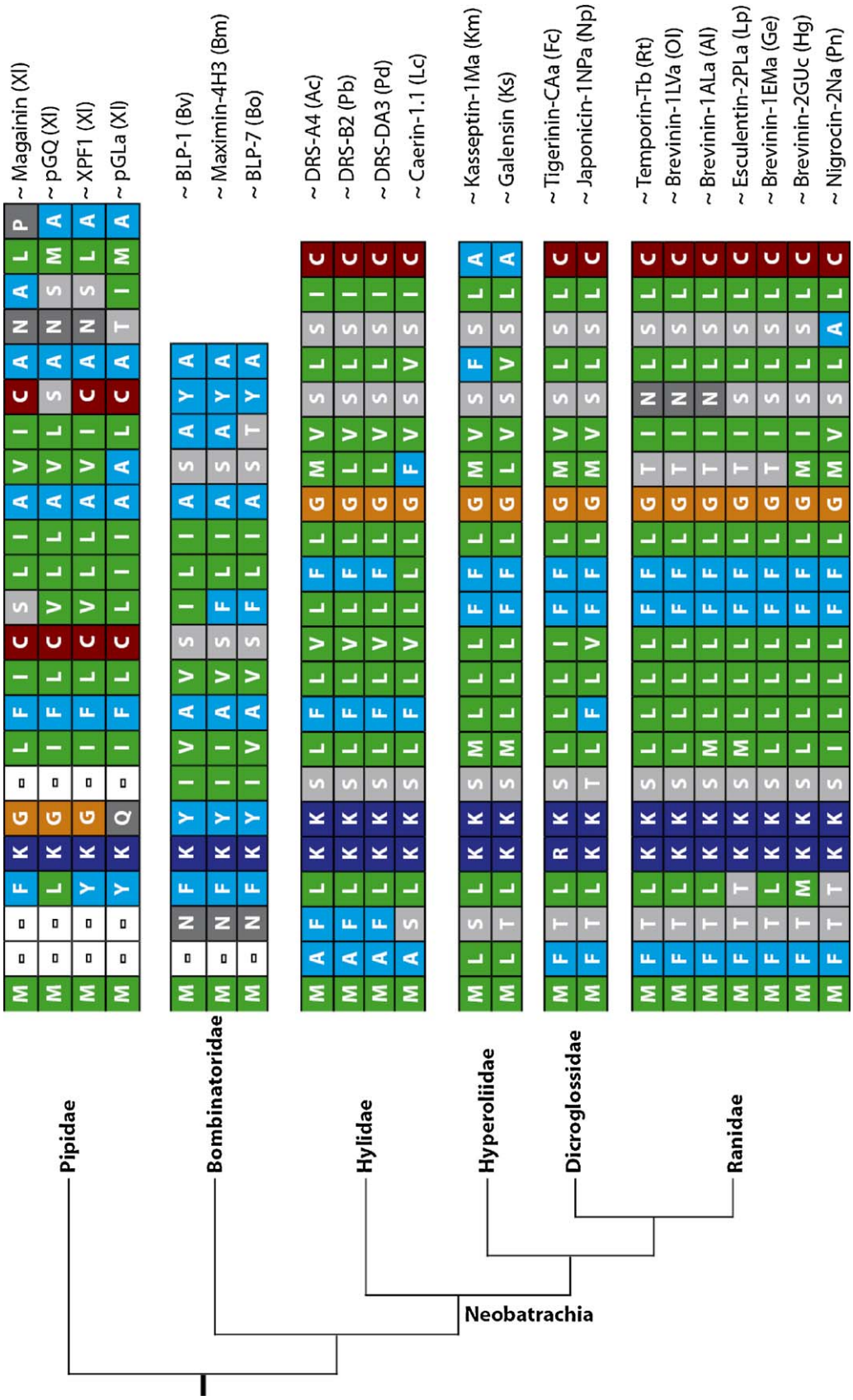

Fig. 1. Alignment of representative AMP signal peptides across Anura. All families for which cDNA sequences of the signal peptides are available are included. All published sequences for *Xenopus laevis* are included, whereas one exemplar sequence per neobatrachian genus and selected sequences for all *Bombina* species investigated are presented. The particular peptides following these signal sequences are indicated. (Ac = *Agalychnis callidryas*; Al = *Amolops loloensis*; Bm = *Bombina maxima*; Bo = *Bombina orientalis*; Bv = *Bombina variegata*; Fc = *Fejervarya cancrivora*; Ge = *Glandirana emeljanovi*; Hg = *Hylarana guentheri*; Km = *Kassina maculata*; Ks = *Kassina senegalensis*; Lc = *Litoria caerulea*; Lp = *Lithobates palustris*; Np = *Nanorana parkeri*; Ol = *Odorrana livida*; Pb = *Phyllomedusa bicolor*; Pd = *Pachymedusa dacnicolor*; Pn = *Pelophylax nigromaculatus*; Rt = *Rana temporaria*; Xl = *Xenopus laevis*).

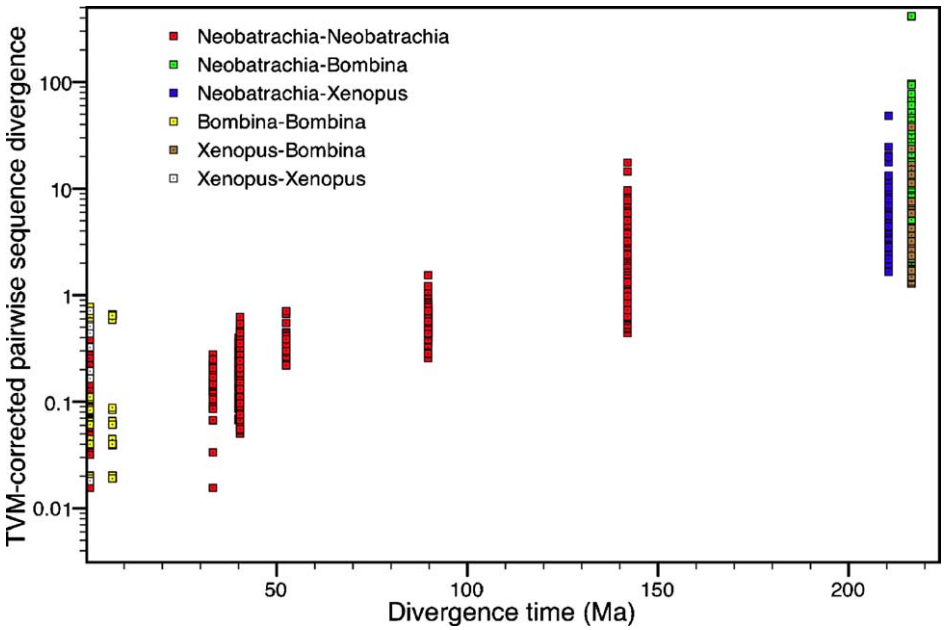

Fig. 2. Relationship between sequence divergence (TVM-corrected distances) and evolutionary time for all pairs of cDNA signal sequences. Comparisons within groups are for Neobatrachia (red), *Bombina* (yellow) and *Xenopus* (white) and between groups are for Neobatrachia versus *Bombina* (green), Neobatrachia versus *Xenopus* (blue), and *Xenopus* versus *Bombina* (brown). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

and *Pachymedusa dacnicolor*, respectively, each of which contains only a single polar residue.

Neobatrachian species are united by having a signal peptide that is 22 amino acids long. The signal sequence is characterized by the double lysine motif Lys-5 and Lys-6 (exception: the single lysine residue Brevinin-1Pb from *Lithobates pipiens*) followed by a polar serine residue (exceptions: Esculentin-1Eb from *Pelophylax esculenta*, Esculentin-1Fa from *P. fukienensis*, Esculentin-1VBb and Esculentin-1VBb from *Odorrana versabilis*, and Esculentin1SCa from *O. schmackeri*). A C-terminal cysteine residue, which might represent a cleavage site for an endoproteolytic peptidase, can be found in all signal peptides except Galensin and Kassinakinin from *Kassina senegalensis* and Dermaseptin-S6 from *Phyllomedusa sauvagei*. In Frenatin-3 and Frenatin-3.1 from *Litoria infrafrenata*, an additional positively charged amino acid (His-2) contributes to the amphipatic nature of the signal peptide. The signal peptide from hyperoliid frogs is highly similar to that of Neobatrachia, although it lacks the C-terminal cysteine residue, which otherwise appears ubiquitous among this motif.

Archaeobatrachian signal sequences are shorter than those from neobatrachians, ranging from 18 amino acids for *Bombina* spp. to 20 for *Xenopus laevis*. All *Xenopus* sequences except PGQ possess two intramolecular cysteines. By contrast, *Bombina* sequences lack cysteine residues, but generally contain two tyrosines (Tyr-5 and Tyr-17). In the Maximin-S signal peptides, Tyr-17 is substituted by a histidine to provide the positive charge that is otherwise missing because these sequences lack the Lys-4 occurring in all other *Bombina* signal sequences. Finally, the *Bombina* Maximin-3H11 and Maximin-8H7 contain an additional basic amino acid residue in position 2 (histidine and lysine, respectively).

3.3. Signal sequence motifs

The alignment of all putative signal peptide sequences (see Fig. 1) shows that a high degree of sequence identity exists among the neobatrachian families Ranidae, Hylidae and Hyperoliidae (average defined pairwise TVM-distance = 0.47; $n = 42{,}374$); among the *Xenopus* sequences (0.37; $n = 10$); and also among the *Bombina* sequences (0.10; $n = 1{,}653$). Between these groups, each of which corresponds to an evolutionary lineage of frogs (Neobatrachia, Pipidae, and Bombinatoridae, respectively), the signal sequences are highly dissimilar (11.4; $n = 1{,}633$). The difference between the average within and between group distances is highly significant ($t = 83.7$; $df = 45{,}668$; $P < 0.0001$).

Furthermore, there is a marked discrepancy in the distribution of "undefined" distances that are greater than would be expected between two completely random sequences. Of the 17,520 total undefined distances (=27.7% of all comparisons), virtually all comprise comparisons between the groups identified above (17,116, or 91.2% of the 18,749 intergroup comparisons). Of the remaining 404 undefined comparisons, all occur between two neobatrachian species and all but three of these between two species that diverged 142 Ma ago.

Finally, no clear relationship exists between the degree of sequence divergence and evolutionary time (Fig. 2), especially when the relationship is extended to include the more ancient between-group comparisons, all of which are 210.5 Ma or older. Over all sequences for which species divergence time information was available, there appears to be a positive log-linear (exponential) relationship ($P = 0$; $df = 22{,}959$; $R^2 = 0.559$). Within Neobatrachia, this tendency is stronger ($P = 0$; $df = 19{,}663$; $R^2 = 0.724$), although a case could also be made for a weak linear relationship ($P = 0$; $R^2 = 0.449$). The latter was not the case for the comparison of all sequences ($P = 3.8 \times 10^{-209}$; but $R^2 = 0.041$). Otherwise, only comparisons within *Bombina* comprised more than a single divergence time and so could be examined using correlation. Although both linear and log-linear relationships were significant ($df = 161$; $P = 0.008$ and 0.009, respectively), both were also extremely weak ($R^2 = 0.004$ for both).

4. Discussion

Antimicrobial peptides have evolved convergently throughout the animal kingdom and show their greatest diversity within anuran amphibians (www.bbcm.univ.trieste.it/~tossi/search.htm7). The traditional explanation for this observation derives from the highly specialized skin of lissamphibians that has to be kept moist for performing physiological functions [22], but, for this very same

reason, is simultaneously susceptible for invading pathogens. Thus, it is generally held that anurans possess a cocktail of AMPs in their skin secretions to actively fight potential "micropredators", thereby completing their efficient armament against natural enemies/macropredators of alkaloids, steroids, biogenic amines and neuroactive peptides.

Our results, however, suggest that the situation is more complex than previously thought. Comparison of all available AMP precursor DNA signal sequences reveals three distinct motifs, each of which is restricted to an evolutionary lineage of frogs (Neobatrachia, Pipidae, and Bombinatoridae), and show little sequence similarity to one another. Furthermore, the pairwise distances of all comparisons scale log-linearly (i.e., exponentially) with evolutionary time. This relationship is driven in part by the highly divergent nature of the oldest comparisons, all of which occur between the three evolutionary lineages. Although comparisons with Neobatrachia also appear to scale log-linearly, removing the oldest comparisons (all 142 Ma) causes linear ($P = 0$; $df = 4{,}151$; $R^2 = 0.752$) and log-linear relationships ($P = 0$; $df = 4{,}151$; $R^2 = 0.773$) to become equally well supported. Finally, the available genomic data does not show any evidence for the neobatrachian and *Bombina* motifs being present within the *Silurana tropicalis* genome. BLAST searches of all DNA signal sequences obtained significant hits for only the *Xenopus* peptides pGQ (identity = 63/65 bp in match domain; $E = 4.0 \times 10^{-24}$), Xenopsin Precursor Fragment (56/60; $E = 2.0 \times 10^{-16}$), Levitide (59/65; $E = 2.0 \times 10^{-14}$), and possibly Magainin (43/50; $E = 0.003$). All other comparisons (surprisingly including the *Xenopus* peptide pGLa), yielded E values of 0.01 or greater with matching domains typically ranging between 17 and 25 bp, the latter suggesting the possibility of one or more common functional domains among the signal peptides.

Thus, we hypothesize that the AMP gene family in anurans has evolved convergently on at least three occasions, mirroring the convergent evolution of this system throughout the animal kingdom. In this context, it is interesting to note that the AMP-encoding gene of the dermaseptin superfamily (from the neobatrachian genus *Phyllomedusa*) also includes neuroactive peptides such as opioids (dermorphins, deltorphins), bradykinin-related peptide [Thr^6]-phyllokinin and tryptophyllin-1 (reviewed in [35]). It has never been contradicted that the two groups of peptides are genetically related due to a considerable extent of signal sequence identity and thus belong to the same gene superfamily although being structurally and functionally distinct [35]. This latter observation hints that AMP genes might possess antipredatory functionality as well as antimicrobial activity in this group. The potential for convergent evolution is also underscored by the fact that cleavage products of histone proteins can also possess antimicrobial activity [32,36], showing that widely different solutions can yield the same functionality. Consequently, we should rather talk about a more general peptide based defense system that is highly adaptive and recruits different genes to cope with the threats present in the actual habitat. This perspective strongly emphasizes the potential for the independent origin of the AMP system, albeit the origin of the two archaeobatrachian AMP gene subfamilies remains unclear.

The alternative explanation of a single, common origin for the three observed motifs seems unlikely, given that the extreme differences between them could only be explained by strong divergent punctuated evolution in the respective lineages. Why this would occur, however, is unclear. The signal sequence is considered to be important for the biology of the expressing cell [35] and is therefore likely under strong functional constraints. If so, the scale of the evolutionary changes would also require a change of the upstream regulatory elements in the expression pathways at the same time. Indeed, signal sequences in Neobatrachia show strong conservation over a period of at least 150 Ma, indicating that long-term stability does occur.

Our hypothesis of convergent evolution does not exclude the possibility for punctuated evolution within each of the three apparent AMP motifs (=gene "suprafamilies"), nor does it conflict with the hypothesis that the hypervariable portions of the AMP genes are under positive selection [42]. For example, the signal peptide of *Kassina senegalensis* lacks the otherwise universally occurring C-terminal cysteine residue that is otherwise typical of the neobatrachian motif and therefore possibly represents a sub-motif within this suprafamily.

5. Conclusion

Testing our hypothesis of the convergent origin of the AMP system in anurans will require greater sampling, both at the genomic and species levels. Because the available *Silurana tropicalis* genome is not complete, it cannot be excluded absolutely that the neobatrachian and *Bombina* signal motifs exist in as yet unsequenced portions of the genome. More importantly, our sampling of AMP genes across Lissamphibia is largely restricted to Neobatrachia. Numerous lineages of "Archaeobatrachia" have yet to be sampled. Recently, *Xenopus amieti* and *X. borealis* have been investigated [14,33], but only on the amino-acid level for the AMPs themselves without information for the signal sequence. Molecular data is also missing for the evolutionary oldest lineage of Ascaphidae [16,12] as well as for *Alytes obstetricans* [13]. Data for AMPs in salamanders and caecilians are scarce and deficient, respectively [25]. However, there is good reason to expect molecules with antimicrobial activity in these latter groups (even if they are not "true" AMP peptides). For instance, removing an adult caecilian from its nest causes molding of all eggs after a few days (Hendrik Müller, pers. comm.). The existence of additional, distinct signal sequence motifs from these underinvestigated groups, as well as for the more divergent neobatrachian lineages, would constitute strong evidence of our hypothesis.

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