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MINI REVIEW

Molecular evolution of animal antimicrobial peptides: widespread moderate positive selection

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Abstract

An increasing number of studies in both vertebrates and invertebrates show that the evolution of antimicrobial peptides is driven by positive selection. Because these diverse molecules show potential for therapeutic applications, they are currently the targets of much structural and functional research, providing extensive background data for evolutionary studies. In this paper, patterns of molecular evolution in antimicrobial peptide genes are reviewed. Evidence for positive selection on antimicrobial peptides includes an excess of nonsynonymous nucleotide substitutions, an excess of charge-changing amino acid substitutions, nonneutral patterns of allelic variation, and functional assays *in vivo* and *in vitro* that show improved antimicrobial effects for derived sequence variants. Positive selection on antimicrobial peptides may be as common as, but perhaps weaker than, selection on the best-known example of adaptively evolving immunity genes, the major histocompatibility complex. Thus, antimicrobial peptides present a useful and underutilized model for the study of adaptive molecular evolution.

Introduction

Determining which genes underlie adaptation, how these genes change in response to natural selection, and what selective pressures drive their evolution are primary goals in the unification of evolutionary biology and genetics. Although many genes under positive natural selection have been identified in particular taxa (Ford, 2002), there are few types of genes for which neutrality is commonly rejected in taxonomically widespread groups. Furthermore, there is often little biochemical knowledge about genes potentially under selection, so the causes of their molecular evolutionary patterns remain largely unknown. Systems of genes regularly under positive selection in diverse species, for which the functional properties of their protein products are well understood, could allow fundamental questions about the genetic basis for Darwinian processes to be addressed.

The identification and characterization of antimicrobial peptides (AMPs) from various taxa is a rapidly expanding field of biology, largely due to the potential for therapeutic applications (Bowman, 2003; Yeaman & Yount, 2003). These peptides are exceptionally diverse in sequence, structure, and function, even among closely related species. Because their diversity is so extensive, and because immune system proteins that interact directly with molecules of pathogens often evolve adaptively (Ford, 2002; Garrigan & Hedrick, 2003), it is likely that much of this variation results from positive selection on the ability to combat new or altered pathogens (Hedengren *et al.*, 2000). Furthermore, because medical and pharmaceutical research is resulting in an increasingly comprehensive understanding of the biochemical properties of these peptides, AMPs may be an ideal system in which to study adaptive molecular evolution.

The AMPs are generally short (usually 15–45 amino acid residues), cationic and amphipathic (Bowman, 2003; Yeaman & Yount, 2003). The mature peptide is often cleaved off of a larger protein containing a signal sequence and a propiece (Bowman, 2003). The occurrence, length and relative position of these three gene regions vary among AMP families (Fig. 1). As it is only the mature peptide that interacts with microbes, selective pressures are likely to differ between this region of the

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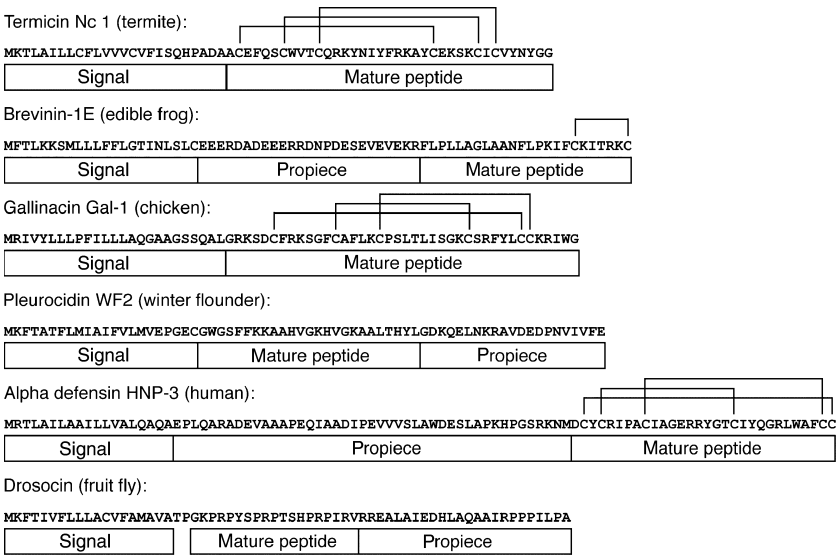

Fig. 1 Examples of antimicrobial peptide genes from various taxa, highlighting structural organization. Disulfide bonds in the mature peptide are shown by connections between cysteines.

gene and the rest of the coding sequence. The structure of the mature peptide varies among gene families. Some AMPs form linear α-helices, while others form β-sheets, and some are composed of an unusually high proportion of particular amino acids (Andreu & Rivas, 1998; Bowman, 2003). Cysteine residues in AMPs often form disulfide bonds important for molecular structure (Fig. 1), thus cysteine codons are expected to be more conserved than other sequence regions. Other modifications such as amidation also occur in some peptides (Andreu & Rivas, 1998).

The mature peptides are active against a wide range of microbes, including bacteria, fungi and viruses. Being positively charged, they bind to anionic microbial lipid membranes and disrupt them through a noncatalytic mechanism. The AMPs may form membrane pores in which the hydrophobic surfaces of the peptides face the hydrophobic membrane core, while the charged surfaces face inward, lining the pore. According to other models, insertion into the hydrophobic membrane core does not necessarily occur, and AMPs simply carpet the membrane until it cannot function (Yeaman & Yount, 2003). The AMPs may also inhibit intracellular functions including DNA or protein synthesis (Andreu & Rivas, 1998; Yeaman & Yount, 2003).

In comparison to the extensive literature describing activity spectra, mechanisms of action, and structural features of AMPs, evolutionary analyses have been rarer. These few studies, though, create a strong case for widespread nonneutral evolution in taxa as diverse as mammals, birds, amphibians, fish and insects (Tables 1 and 2). Most evolutionary change and variation in genes is neutral, with selection serving primarily as a conservative force, so genes at which positive selection is driving evolution are unusual and show unique signatures. In this paper, studies of the molecular evolution of AMPs are reviewed, with a focus on evidence for positive selection acting on these molecules.

Evolutionary relationships

It is unknown whether all antimicrobial peptides are homologous. Neither sequence nor structure is conserved among gene families, and a reasonable hypothesis is that small peptides with antimicrobial abilities have evolved multiple times. Nevertheless, similar evolutionary pressures may be acting on unrelated families of AMPs, and they may show similar signatures of these forces.

Evolutionary relationships are not always clear within gene families, either. Any analysis of molecular evolution at AMP loci is complicated by the fact that these genes are frequently duplicated. For example, the mouse genome contains 49 β-defensin loci, in addition to other AMPs (Schutte *et al.*, 2002). In humans, a cluster of three β-defensin genes varies in copy number from 2 to 12 repeats per diploid genome (Hollox *et al.*, 2003). The frequent duplication of AMP genes may itself be adaptive, allowing new copies to develop novel antimicrobial properties without constraint to retain old functions. In general, it can be assumed that a gene's most similar homolog in a different species is an ortholog, although the true ortholog may have been deleted or gone undetected. Conversely, homologous sequences from the same species may be reported to be paralogs (e.g. Duda *et al.*, 2002), when they may in fact be different alleles of the same locus. An added complication is that all regions of a gene may not share the same evolutionary history due to exon shuffling (Froy & Gurevitz, 2003).

Confidence of orthology is especially important in tests of selection based on intraspecies variation, where, for example, unknowingly including sequences from multiple loci can lead to erroneous conclusions. Tests of

Table 1 AMP families that show evidence of positive selection in some sequence comparisons (not necessarily over all available sequences).

AMP family	Taxon	Evidence of selection over all codons	Site heterogeneity
α-Defensin†,‡,†††††,‡‡‡‡‡	Primates, rodents, rabbits	dn/ds >1*, radical/conservative change >1*	dn/ds >1* at certain codons
β-Defensin‡,§,††,‡‡,§§,¶¶,¶¶¶,§§§§	Primates, rodents, bovids	dn/ds >1*, radical/conservative change >1*	dn/ds >1* at certain codons
Gallinacin¶¶¶¶,§§§§§	Chickens	dn/ds >1	dn/ds >1* at certain codons
Frog AMP¶,†††,‡‡‡,††††,¶¶¶¶¶	Ranid and hylid frogs	dn/ds >1*, radical/conservative change >1*	Not tested
Pleurocidin¶¶¶¶¶	Flatfishes	dn/ds >1, radical/conservative change >1	dn/ds >1* at certain codons
Termicin‡‡‡‡	Termites	dn/ds >1, radical/conservative change >1	dn/ds >1* at certain codons
Ceratotoxin§§§	Fruit flies	dn/ds >1 (very rarely)	Not tested

*Statistically significant; †Hughes & Yeager (1997); ‡Hughes (1999); §Del Pero *et al.* (2002); ¶Duda *et al.* (2002); ††Boniotto *et al.* (2003a); ‡‡Boniotto *et al.* (2003b); §§Maxwell *et al.* (2003); ¶¶Morrison *et al.* (2003); †††Nicolas *et al.* (2003); ‡‡‡Pupko *et al.* (2003); §§§Rosetto *et al.* (2003); ¶¶¶Semple *et al.* (2003); ††††Vanhoye *et al.* (2003); ‡‡‡‡Bulmer & Crozier (2004); §§§§Luenser & Ludwig (2005); ¶¶¶¶Lynn *et al.* (2004a); †††††Lynn *et al.* (2004b); ‡‡‡‡‡Patil *et al.* (2004); §§§§§Xiao *et al.* (2004); ¶¶¶¶¶Tennessen, unpublished.

selection based on substitution rate ratios do not depend on assumptions about orthology. However, the absolute divergences at both synonymous and nonsynonymous sites are dependent on the time since the sequences last shared a common ancestor, which can only be estimated with knowledge of orthology. In addition, identification of orthologs and paralogs allows one to address other issues, such as whether selection is strongest on recently duplicated genes.

Nonsynonymous and synonymous substitution rates

If the ratio of nonsynonymous nucleotide differences (dn) to synonymous nucleotide differences (ds) between sequences is significantly greater than one, positive selection can be inferred (Hill & Hastie, 1987; Hughes & Nei, 1988). The dn/ds ratio is often greater than one for some AMP families in both vertebrates and invertebrates (Table 1). Most comparisons have been between paralogs, but a few apparent β-defensin orthologs show significantly high dn/ds between species of mouse (Morrison *et al.*, 2003), and between species of primate (Boniotto *et al.*, 2003b).

Such examples are noteworthy, but it is important to realize that for many pair-wise comparisons in these AMP families, dn/ds is not significantly different from, or is even less than, one. In addition, for several other families of AMPs, dn/ds is consistently less than one (e.g. β-defensin 1, Del Pero *et al.*, 2002; β-defensin 3, Boniotto *et al.*, 2003a). Among fruit fly AMPs, dn/ds greater than one has only been observed in a small minority of comparisons from tephritid flies (Rosetto *et al.*, 2003); in *Drosophila*, this ratio approaches, but has not been observed to exceed, one. A deficiency of nonsynonymous substitutions does not rule out selection, though, because the dn/ds test is very conservative. In almost any coding sequence, purifying selection is acting on many of the codons, which can lower the value of dn even in the face of positive selection on other codons. Thus, by using maximum likelihood methods that test dn and ds at individual codons (Yang & Bielawski, 2000), significant positive selection on portions of AMP sequences has been detected in many gene families (Table 1). Cysteines are highly conserved and are not under positive selection, but other sites near them in the mature peptide region are frequently positively selected.

Several authors have noticed that dn/ds ratios in AMPs are highest when divergence is low (Hughes & Yeager, 1997; Hughes, 1999; Morrison *et al.*, 2003; Nicolas *et al.*, 2003; Semple *et al.*, 2003). This pattern, in which selection appears the strongest between closely related genes, could be due to intense selection immediately after gene duplication that eventually diminishes after the duplicated gene has adapted to its new function. It is also possible that nonsynonymous sites at which positive selection tends to act are more saturated at greater genetic distances, limiting the observed dn in highly divergent sequences.

As noted above, the translated protein usually consists of a signal sequence, a propiece, and the mature peptide that is cleaved off. In vertebrates, the mature peptide evolves faster than the rest of the protein (Hughes & Yeager, 1997; Duda *et al.*, 2002; Maxwell *et al.*, 2003; Morrison *et al.*, 2003; Semple *et al.*, 2003; Xiao *et al.*, 2004). Although such rapid evolution may be due to a relaxation of purifying selection relative to the rest of the sequence, it implies that, if positive selection is indeed occurring, it is acting on the mature peptide. Some insect AMPs, such as termite termicins (Bulmer & Crozier, 2004) and andropin in *Drosophila* (Date-Ito *et al.*, 2002) follow the vertebrate pattern of greater amino acid divergence in the mature peptide than in the signal sequence. Other *Drosophila* AMPs, however, show the opposite pattern: the mature peptide itself is highly conserved, and positive selection, if it is acting, is focused on the other parts of the protein or on regulatory sequences (Lazzaro & Clark, 2001,2003).

Interestingly, even silent sites at several AMP loci show unusually high divergence in the mature peptide region with respect to other regions of the same gene. For example, synonymous divergence among rabbit

Table 2 AMP families assessed for allelic variation in nucleotide sequences.

AMP family	Taxon	Evidence of nonneutrality
β-Defensin‡,**,‡‡,§§,¶¶,†††	Humans	Disease resistance correlation
Andropin*,††	Fruit flies	HKA test, Fu and Li test
Drosocin***	Fruit flies	Fay and Wu's H, rapid allele frequency change
Diptericin*,***,‡‡‡	Fruit flies	Fay and Wu's H, Fu and Li test, disease resistance correlation
Attacin¶,***,‡‡‡	Fruit flies	Fay and Wu's H, disease resistance correlation
Metchnikowin***,‡‡‡	Fruit flies	Fay and Wu's H, epistatic disease resistance correlation
Defensin***,‡‡‡	Fruit flies	Epistatic disease resistance correlation
Cecropin*,†,§,***	Fruit flies	None

*Clark & Wang (1997); †Date *et al.* (1998); ‡Dörk & Stuhrmann (1998); §Ramos-Onsins & Aguadé (1998); ¶Lazzaro & Clark (2001); **Vatta *et al.* (2000); ††Date-Ito *et al.* (2002); ‡‡Jurevic *et al.* (2002); §§Matsushita *et al.* (2002); ¶¶Jurevic *et al.* (2003); ***Lazzaro & Clark (2003); †††Braida *et al.* (2004); ‡‡‡Lazzaro *et al.* (2004).

defensins is significantly higher in the propiece and mature peptide then in the signal sequence (Hughes & Yeager, 1997). A similar trend has been recorded in amphibian AMPs (Nicolas *et al.*, 2003; Vanhoye *et al.*, 2003; Tennessen, unpublished). Furthermore, ds in the flatfish pleurocidin mature peptide region is significantly higher than both ds in the signal sequence and divergence in the introns of the gene (Tennessen, unpublished). The difference between synonymous divergence in the signal sequence and mature peptide is especially unexpected in the pleurocidins, because part of the mature peptide is on the same exon as the signal sequence, immediately adjacent to it. Thus, the mechanism causing enhanced synonymous divergence is extremely specific to the mature peptide region. All gene families exhibiting enhanced synonymous divergence in the mature peptide region also show evidence of positive selection on that part of the gene. This widespread trend may be caused by an increased mutation rate, suggesting that vertebrates promote variation in a gene region where it is adaptive to do so. Alternatively, selection on 'silent' sites, perhaps for translational accuracy or mRNA structure, may be the explanation.

In similar but not identical pattern, the *D. melanogaster* cecropin and attacin gene regions show significantly high levels of silent nucleotide variation relative to other loci in that species (Date *et al.*, 1998; Lazzaro & Clark, 2001). The authors hypothesized that intragenic recombination and/or introgression from a related species could have caused the excess of synonymous variation, but the hypotheses described above for the pattern seen in vertebrate AMPs could also be responsible. There are no reports of a difference between silent sites in the mature peptide and the rest of the coding region in *Drosophila*.

Patterns of amino acid change

Nonsynonymous substitutions that replace an amino acid residue with a residue of a different charge can be defined as radical, and an excess of radical nonsynonymous differences between sequences can be taken as evidence for positive selection (Zhang, 2000; Pupko *et al.*, 2003). In the mature peptide, nonsynonymous changes that are radical with respect to charge are sometimes significantly more frequent than conservative nonsynonymous changes (Table 1). Some AMPs evolve conservatively with respect to charge, such as those from ranid frogs (Duda *et al.*, 2002), but in general change in charge is surprisingly common. The termicins from termites in particular are noteworthy in that selection appears to have acted in several independent lineages to reduce net charge (Bulmer & Crozier, 2004). Although radical nonsynonymous changes can also be defined as changes in polarity or volume, AMP evolution is rarely radical by these definitions. As positive charge is thought to be important in antimicrobial activity (Andreu & Rivas, 1998), it is notable that charge is not a conserved feature. Rather, subtle differences in net charge or the location of charged residues apparently convey higher fitness on a frequent basis.

It has been proposed that the anionic propiece of the processed domain interacts with the cationic mature peptide and prevents it from damaging the host cells (Michaelson *et al.*, 1992). In mammalian α-defensins, the propiece and the mature peptide evolve in a coordinated manner such that charge changes in the propiece tend to be accompanied by opposite charge changes in the mature peptide, resulting in little change in net charge (Hughes & Yeager, 1997). A similar hypothesis was proposed for hylid frog AMPs (Duda *et al.*, 2002) but an alternate analysis suggests that coordinated evolution does not occur in this group (Tennessen, unpublished). No evidence of coordinated evolution was seen in ranid frog AMPs, mammalian β-defensins, or flatfish pleurocidins (Duda *et al.*, 2002; Morrison *et al.*, 2003; Tennessen, unpublished). Thus, coordinated evolution may be unique to mammalian α-defensins, and not a general feature of AMPs.

Allelic variation

Intraspecies variation at AMP loci has been assessed in *Drosophila* (Clark & Wang, 1997; Date *et al.*, 1998; Ramos-Onsins & Aguadé, 1998; Lazzaro & Clark, 2001,2003; Date-Ito *et al.*, 2002) and humans (Dörk & Stuhrmann, 1998; Vatta *et al.*, 2000; Jurevic *et al.*, 2002) (Table 2). This variation often has phenotypic effects, which could be subject to selection. For example, polymorphisms in both coding and noncoding regions of the human β-defensin 1 gene have been correlated with susceptibility to diseases such as oral fungal infection, chronic obstructive pulmonary disease, and HIV-1 infection (Matsushita *et al.*, 2002; Jurevic *et al.*, 2003;

Braida *et al.*, 2004). Likewise, in *Drosophila*, variation at some AMP loci shows an association with phenotypic variability in pathogen resistance, and variation at several other loci affects disease resistance through epistatic interaction with intracellular signalling loci, probably due to variation in transcriptional regulation (Lazzaro *et al.*, 2004) (Table 2). In some of these cases, the allele conveying higher fitness is the ancestral sequence, which is consistent with purifying, not positive, selection. In other cases, the opposite is true. For example, a derived variant of the β-defensin 1 5′-UTR in humans is significantly associated with lower oral fungal infection, which arguably could convey higher fitness and thus be under positive selection (Jurevic *et al.*, 2003).

True tests of neutrality using intraspecies variation have only been reported in *Drosophila*. In some regards this variation behaves neutrally. For example, a significant excess of nonsynonymous variation has not been observed at any locus. McDonald & Kreitman (1991) tests on cecropin, andropin and diptericin loci have failed to reject neutrality (Clark & Wang, 1997; Date-Ito *et al.*, 2002). The cecropin family in particular shows no evidence of positive selection despite extensive study (Clark & Wang, 1997; Date *et al.*, 1998; Ramos-Onsins & Aguadé, 1998; Lazzaro & Clark, 2003).

Other tests show nonneutral patterns in *Drosophila* allelic variation (Table 2). For example, a significant excess of high-frequency derived variants, as demonstrated by Fay & Wu (2000) H-tests, has been noted at several loci (Lazzaro & Clark, 2001,2003). A propiece variant of drosocin appears to 'have substantially increased in frequency in only 2 years' (Lazzaro & Clark, 2003). In an application of the HKA test (Hudson *et al.*, 1987), andropin exhibits a significantly high ratio of divergence to polymorphism when compared to cecropin B, which conforms to neutral patterns (Clark & Wang, 1997). Finally, Fu & Li (1993) tests gave a significantly negative result for two loci (Clark & Wang, 1997), which is probably due to purifying selection but could potentially indicate recovery from a positive selective sweep.

Antimicrobial activity and selection

Because most studies of AMPs focus on their capacity to kill pathogens, there is a great deal of evidence that AMPs vary considerably in their antimicrobial activities (Tossi *et al.*, 2000; Yeaman & Yount, 2003). Diversity in antimicrobial activity among closely related AMPs is consistent with the hypothesis that their evolutionary radiation has not been merely a neutral drift to variants of equivalent fitness. Derived variants sometimes show improved antimicrobial activities, which further confirms the hypothesis of positive selection. For example, in the northern leopard frog *Rana pipiens*, the derived AMP brevinin-1Pc is nearly three times more efficient at killing *E. coli* than the more basal AMPs brevinin-1Pa and brevinin-1Pb (Goraya *et al.*, 2000; Conlon *et al.*, 2004). In addition, variation in antimicrobial activities within adaptively evolving gene families can provide insight into the selective pressures acting on these AMPs. For example, in a primate phylogeny, a significant excess of nonsynonymous change in the β-defensin 2 gene was detected along the branch leading to humans from the common ancestor of humans and macaques (Boniotto *et al.*, 2003b). Counterintuitively, macaque β-defensin 2 appears more lethal towards some human pathogens than the positively selected human β-defensin 2 (Antcheva *et al.*, 2004). This result suggests either that these microbes were not the ones driving selection, or that the microbes themselves have evolved a greater tolerance towards human AMPs.

Causes of positive selection

Although individual tests of selection on AMPs are sometimes merely suggestive, taken together these studies present convincing evidence that positive selection on AMPs is common and taxonomically widespread. Positive selection is not constant in all lineages at all times, as some evolutionary change in AMPs appears to be neutral. In addition, even where positive selection does occur, it may not be very strong. Nevertheless, there are few examples of gene types for which neutrality is rejected as frequently as for AMP genes, suggesting that these molecules are highly important to adaptive evolution. Clearly, the innate immune system is not merely a vestigial relic. Rather, new AMP variants are frequently able to provide higher fitness to both vertebrates and invertebrates.

Why does selection appear to act differently on different AMP loci, given that they all have similar functions? Is it simply an artifact of the statistical tests used? If not, are there fundamental differences in the roles of these molecules? Or, do most or all of them alternate between periods of neutral evolution and periods of positive adaptation, with researchers arbitrarily happening to observe them in one period or the other? The specific pathogens driving selection undoubtedly vary among hosts, which could result in different patterns of evolution. Some hosts might be in a constant co-evolutionary arms race with pathogens that are under selection to resist their defenses. A variety of resistance mechanisms to AMPs are known in microbes, some of which involve a single gene product (Andreu & Rivas, 1998; Yeaman & Yount, 2003). In some cases, derived microbial strains are more resistant than the wild type (Fernandez & Weiss, 1996; Thevissen *et al.*, 2004), which is consistent with the hypothesis that positive selection on microbial genomes can result in increased resistance to AMPs. Thus, it may be the case that resistance is easy to evolve and happens frequently. On the other hand, some AMPs might attack their targets in such a way that evolving resistance is not possible without coordinated changes at many microbial genes. Selection on these

AMPs would primarily occur when hosts enter new niches and are forced to adapt to completely different pathogen species not previously encountered.

Comparisons with MHC

Genes of the immune system, especially those that interact directly with molecules of pathogens, have been shown to evolve nonneutrally more often than many other types of genes (Ford, 2002; Garrigan & Hedrick, 2003). The best-studied example is the Major Histocompatibility Complex (MHC), which encodes glycoproteins that bind foreign peptides for pathogen recognition. It has been shown to deviate from neutral expectations at population, species, and/or interspecies levels in multiple vertebrate taxa and is one of the clearest examples of a locus complex that frequently evolves adaptively (Hughes & Yeager, 1998; Garrigan & Hedrick, 2003). Having a similar but not identical function, AMPs might be expected *a priori* to evolve nonneutrally, albeit perhaps in different ways than genes such as MHC loci, and it is worth comparing signatures of selection on AMPs with those on other parts of the immune system.

Positive selection in both MHC and AMP loci is common, but not universal. As in AMPs, comparisons of MHC sequences among related species sometimes, but not always, show a dn/ds ratio significantly higher than one (Bernatchez & Landry, 2003; Garrigan & Hedrick, 2003). The AMPs are more taxonomically widespread than MHC, which only occurs in vertebrates. In the species that share both types of genes, an obvious question is whether nonneutral evolution is more frequent in MHC or in AMPs. Although MHC has received more attention, positive selection on AMPs may be equally common. If so, their occurrence in both vertebrates and invertebrates, as well as in plants, would make AMPs a more general model system for the study of adaptive molecular evolution.

Positive selection for new variants in MHC is often accompanied by the maintenance of multiple allelic lineages via extremely strong balancing selection. Evidence for balancing selection in MHC includes uniform allele frequencies, an excess of nonsynonymous variation, and transspecies polymorphisms in which sequences are more similar to orthologous sequences in other species than they are to alleles at the same locus in the same species (Bernatchez & Landry, 2003; Garrigan & Hedrick, 2003). Like AMPs, MHC sometimes appears to have an elevated synonymous substitution rate relative to adjacent sequences (Hughes, 2000). In this case, excess synonymous variation is attributed to selection: ancient MHC sequences have been maintained by balancing selection longer than neutral drift would allow, which has let many synonymous substitutions accumulate (Hughes, 2000).

In contrast with MHC, there is little direct evidence for balancing selection on AMPs. Intraspecies variation, in the few AMP loci where it has been assessed, is not as excessive as in MHC (Clark & Wang, 1997). Neither unusually uniform allele frequencies nor an excess of nonsynonymous variation over synonymous variation has been reported in these studies, either. However, such studies are rare, and include some of the AMPs that show the least evidence for selection at the interspecies level, such as β-defensin 1 in humans and cecropin in *Drosophila*. While transspecies polymorphisms of AMPs have not been shown to occur, either, sequences from the same species sometimes do not cluster together in evolutionary trees (e.g. Conlon *et al.*, 2004). Although such a phylogenetic pattern is likely due to gene duplication, a clear understanding of orthology and paralogy is usually lacking. Balancing selection could enhance synonymous site diversity among alleles at a locus, and the subsequent separation of these divergent alleles during a speciation or gene duplication event would lead to the observed enhanced synonymous site divergence of the descendant sequences. However, for balancing selection to cause the pattern of enhanced synonymous divergence in the mature peptide region relative to the signal sequence, these two gene regions would have to be unlinked, which may not always be the case, especially for the pleurocidins. Thus, the possibility remains that balancing selection acts on at least some AMP loci, but lacking further evidence the null hypothesis of no balancing selection must be retained.

Whether selection on AMPs is balancing or simply directional, it may be generally weaker than the selection acting on MHC. It is difficult to evaluate this hypothesis directly. Tests of MHC evolution do not always reject neutrality, and those that do reject it estimate selection coefficients ranging over several orders of magnitude (Satta *et al.*, 1994; Garrigan & Hedrick, 2001; Aguilar *et al.*, 2004). The MHC evolution has been much more extensively studied than AMP evolution, making direct comparisons of selection estimates difficult. However, as AMPs appear to lack some of the extraordinary patterns exhibited by MHC, such as its extreme polymorphism, an appropriate null hypothesis is that AMPs evolve more neutrally than MHC. In addition, the fact that AMPs are not thought to recognize specific molecules the way that MHC does suggests that selection on AMPs should not be as strong as on MHC. Within certain motifs, all AMP variants might have equal antimicrobial ability, such that selection is weaker than if every difference mattered.

Future directions

The AMPs provide an opportunity to study adaptation at the level of the molecules where it actually happens. Specific selective pressures driving evolution in AMPs could be investigated by identifying how positively selected changes affect antimicrobial activity. In addition, research could aim to elucidate both sides of a coevolutionary arms race between host and pathogen by

identifying the changes in microbial genes that cause resistance to AMPs. Further comparisons between AMPs and MHC would also be interesting, particularly, of the strength and frequency of selection and whether selection at AMP genes is sufficient to overcome the effects of drift in small populations, as has been shown for MHC (Aguilar *et al.*, 2004; Jarvi *et al.*, 2004). More research at the population genetic level may be able to address many of the unresolved issues in AMP evolution, including specific selective pressures, strength of selection on AMPs vs. MHC, and the cause of enhanced synonymous divergence. Those searching for therapeutic uses of AMPs should seek to identify AMP families in which selection has likely produced high variation in antimicrobial properties, as well as AMP families that do not appear to be under constant selection to change, perhaps because pathogens cannot easily evolve resistance to them. On the other hand, some proposed uses for AMPs, such phylogenetic analysis (Conlon *et al.*, 2004), may not be feasible if nonneutral evolution is common. Continued investigation into AMP evolution promises to be a highly fruitful and fascinating area of research.

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