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ORIGINAL ARTICLE

Characterization of the antimicrobial peptide family defensins in the Tasmanian devil (*Sarcophilus harrisii*), koala (*Phascolarctos cinereus)*, and tammar wallaby (*Macropus eugenii)*

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Abstract Defensins comprise a family of cysteine-rich antimicrobial peptides with important roles in innate and adaptive immune defense in vertebrates. We characterized alpha and beta defensin genes in three Australian marsupials: the Tasmanian devil (*Sarcophilus harrisii*), koala (*Phascolarctos cinereus*), and tammar wallaby (*Macropus eugenii*) and identified 48, 34, and 39 defensins, respectively. One hundred and twelve have the classical antimicrobial peptides characteristics required for pathogen membrane targeting, including cationic charge (between 1+ and 15+) and a high proportion of hydrophobic residues (>30%). Phylogenetic analysis shows that gene duplication has driven unique and species-specific expansions of devil, koala, and tammar wallaby beta defensins and devil alpha defensins. Defensin genes are arranged in three genomic clusters in marsupials, whereas further duplications and translocations have occurred in eutherians resulting in four and five gene clusters in mice and humans, respectively. Marsupial defensins are generally under purifying selection, particularly residues essential for defensin structural stability. Certain hydrophobic or positively charged sites, predominantly found in the defensin loop, are positively selected, which may have functional significance in defensin-target interaction and membrane insertion.

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Introduction

Defensins are a family of cysteine-rich polypeptides that play critical roles in innate and adaptive immune defense (Yang et al. 1999) in vertebrates (Lehrer and Ganz 2002; Ganz 2004), invertebrates (Hoffmann and Hetru 1992; Lehrer and Ganz 1999; de la Vega and Possani 2005), and plants (Broekaert et al. 1995). These include defense against bacterial (Lehrer et al. 1989; Schibli et al. 2002), fungal (Edgerton et al. 2000; Feng et al. 2005) and viral pathogens (Daher et al. 1986), immunomodulatory functions (Bowdish et al. 2006; Grigat et al. 2007; Steinstraesser et al. 2011), roles in reproduction and fertility (Zhou et al. 2004; Patil et al. 2005; Narciandi et al. 2011; Tollner et al. 2011), and natural flora control (Salzman et al. 2007). Defensin characterization is critical in defining a species' host-defense peptide repertoire and understanding its immune system. Defensins are also ideal for studying adaptive molecular evolution because of their intrinsic link with rapidly evolving pathogens (Hughes 1999; Semple et al. 2003; Tennessen 2005; Cheng et al. 2015). The antimicrobial affinity of defensins makes them promising templates for future classes of novel antibiotics and in vivo gene therapy (Huang et al. 2002; Thevissen et al. 2007; Easton et al. 2009), which are urgently required to combat multidrug-resistant pathogens.

Three defensin subfamilies, alpha (α), beta (β), and theta (θ), have been described in mammals, with alpha and beta genes found in all lineages (Ganz 2004). Defensins typically consist of a conserved signal sequence and a propeptide sequence encoded by the first one to two exons, and a mature peptide domain encoded by the terminal exon (Ouellette and Selsted 1996; Ghosh et al. 2002). They are initially synthesized

as precursor molecules which are post translationally processed to form an active mature peptide (Valore and Ganz 1992). Intramolecular disulfide bridges form between three cysteine pairs in mature peptides, defining the subfamilies: disulfide bond bridge cysteines 1–6, 2–4, and 3–5 in alpha defensins and cysteines 1–5, 2–4, and 3–6 in beta defensins (Ganz et al. 1985; Selsted et al. 1985).

The gray short-tailed opossum (*Monodelphis domestica*) is the only marsupial in which defensins have been characterized (Belov et al. 2007). Australian marsupials diverged from American marsupials 80 million years ago (MYA) (Meredith et al. 2008). The Tasmanian devil (*Sarcophilus harrisii*), koala (*Phascolarctos cinereus*), and tammar wallaby (*Macropus eugenii*) last shared a common ancestor 60 MYA, representing three distinct lineages within the Australasian radiation (Phillips et al. 2006; Meredith et al. 2008). The Tasmanian devil and koala have both been severely affected by contagious diseases in recent years (McCallum et al. 2007; Rhodes et al. 2011). The devil faces extinction due to devil facial tumor disease (Lachish et al. 2007), while the koala faces an ongoing battle with chlamydiosis and the koala retrovirus (KoRV) (Polkinghorne et al. 2013). Characterization of defensins in these species is the first step in defining their role in host health and will inform our understanding of the evolution of this important peptide family. Here, we decribe alpha and beta defensins in the Tasmanian devil, tammar wallaby, and koala and discuss their evolution.

Methods

Computational search for defensin genes

Known opossum, platypus, human, and mouse defensin sequences were used to perform BLASTN and BLASTP (Altschul et al. 1997) searches against the devil (Murchison et al. 2012), tammar wallaby (Renfree et al. 2011), and koala (KGC, unpublished data) genomes. For evidence of transcription and to refine genome annotations, BLASTP and TBLASTN searches were performed on published transcriptome data from devil (Murchison et al. 2012) (Hewavisenti et al. 2016), tammar wallaby (Wong et al. 2011), and koala (Hobbs et al. 2014) (Supplementary Table 1). Significant BLAST hits (e-value $<10^{-5}$; identity over 60%) were retrieved and aligned with CLUSTALW (Thompson et al. 1994) and examined for the defensin cysteine motif or the conserved signal sequence in Bioedit 7.0.0 (Hall 1999). Novel defensin sequences were then used as the query sequences for additional BLASTN and BLASTP searches. Seven hidden Markov models (HMMs) were generated using HMMER 3.0 (Finn et al. 2011): four targeted the six cysteine mature peptide motif and three targeted the first exon signal sequence. Representative opossum, human, mouse, and dog defensin sequences were used to create the initial HMMs, which were then updated to include novel Australian marsupial defensin sequences with HMMER searches repeated. All HMMER searches were performed on a six frame translation of each marsupial genome. Based on previously defined defensin intron lengths (Patil et al. 2005), signal sequences within 15 Kbp upstream of a mature peptide motif were predicted to be the matching signal sequence. Defensins were named sequentially as they were identified using an abbreviated species name, a subfamily suffix, and a gene number.

Phylogenetic analysis

Alpha and beta defensin peptide sequences were each aligned using CLUSTALW. A beta defensin tree was constructed using 41 devil, 32 koala, 36 wallaby, 46 opossum, 36 human, and 46 mouse sequences, and the alpha defensin tree consisted of seven devil, two koala, three wallaby, two opossum, five human, six chimpanzee, 13 mouse, and 11 rat sequences (Supplementary Tables 2 & 3). Phylogenetic trees were constructed using the neighbor-joining method based on p-distance of aligned amino acids in MEGA 5 (Tamura et al. 2011). A thousand bootstrap replicates were used to test phylogeny reliability (Felsenstein 1985).

Synteny analysis

Synteny groups were based on genomic organization and phylogenetic analysis of orthologs between species. The ENSEMBL genome browser (Release 84) (Cunningham et al. 2015) was used to determine the position and orientation of human and mouse defensin genes according to the latest human (Genbank assembly ID GCA_000001405.20 released 2014) and mouse (GenBank Assembly ID GCA_000 001635.6 released 2012) genome assemblies. Human and mouse defensin cluster flanking genes were used as query sequences to BLAST the Tasmanian devil, koala, wallaby, and opossum genomes to determine their location and orientation (Supplementary Table 2). Wallaby was excluded from synteny analysis due to the fragmented nature of the genome assembly.

Selection tests

The data monkey web server (Pond and Frost 2005a; Delport et al. 2010) was used to assess individual residues under positive and negative selection. Fixed Effect Likelihood (FEL) (Pond and Frost 2005b) and Fast Unconstrained Bayesian Approximation for Inferring Selection (FUBAR) (Murrell et al. 2013) were used to detect both negatively and positively selected sites. Mixed Effects Models of Evolution (MEME) (Murrell et al. 2012) was also used to test positively selected sites. Significance value for FEL and MEME were set at

$p < 0.05$, and posterior probability for FUBAR was >0.9. Overall nucleotide substitution rates in the first and second exon were calculated using the Kumar method (Nei and Kumar 2000) in MEGA 5. Due to defensin cleavage site variability, N-terminus of the putative mature beta defensin was trimmed at four amino acids prior to the first cysteine of the 6-cysteine motif; alpha defensin mature peptide start site was predicted as the first trypsin cleavage site (arginine: R or lysine: K) prior to the first cysteine.

Defensin sequence characterization

The overall hydrophobicity percentage of each defensin was calculated using an antimicrobial peptide database (Wang and Wang 2004). Hydrophobic residues are defined according to the Kyte-Doolittle scale (Kyte and Doolittle 1982). Peptide sequence logos were created for each defensin subfamily using WebLogo (Crooks et al. 2004) and were based on alignments of all Tasmanian devil, koala, and tammar wallaby defensin sequences identified. The absolute site variability using the Wu-Kabat variability coefficient (Kabat 1969) was used to generate a variability plot (Garcia-Boronat et al. 2008) using the same sequence alignments used for the selection tests and web logo generation. Peptide charge was calculated using pKa values of negatively and positively charged residues taking into account disulfide bonds, as previously described (Cheng et al. 2015).

Results and discussion

Defensin gene annotation

Forty-one beta defensins and seven alpha defensins were identified in the genome of the Tasmanian devil; 33 beta defensins and two alpha defensins were identified in the koala genome; and 36 beta defensins and three alpha defensins were identified in the tammar wallaby genome. Due to the fragmented nature of these genome assemblies, 70 of the 121 putative defensins identified were not assigned a first exon signal sequence and therefore may include some pseudogenes (Supplementary Table 2).

Characteristics of marsupial defensins

Intron length varied considerably in the annotated defensin genes, with the shortest spanning 99 bp (SahaDefB26) and the longest 14,844 bp (PhciDefB29); the mean intron length was 2 Kbp. Based on transcriptome data available, 10 devil defensins and nine koala defensins were transcribed (Supplementary Table 2). One defensin had a third exon encoding a short prosegment piece. As our HMMs targeted signal sequences and mature peptides only, some other defensins may also have prosegment pieces encoded by third exons, which will require transcript data to identify. The mature peptide length varied between 40 and 140 amino acids in beta defensins and between 30 and 35 amino acids in alpha defensins. Mature defensins are usually shorter than 50 amino acids (Ganz 2003). Large beta defensins with elongated C-terminal tails, like the ones we found in marsupials, have also been described in mice, yet the biological function or potential cleavage of these tails is unknown (Schutte et al. 2002).

Classically, mammalian defensins have a conserved 6-cysteine mature peptide motif. The majority of marsupial defensins identified exhibit this motif, though we also observed a few defensin-like variants, including three 8-cysteine alpha variants (SahaDefA7, MaeuDefA3, PhciDefA2), three 5-cysteine beta variants (SahaDefB41, PhciDefB23, MaeuDefB29), and three 7-cysteine variants (SahaDefA4, SahaDefB31, PhciDefA1). The 8-cysteine variant in the devil (SahaDefA7) was transcribed in the devil facial tumor and the one in koala (PhciDefA2) was transcribed in multiple tissues (Supplementary Table 2). Disulfide bridges in defensins assist in peptide stabilization; facilitate target membrane interaction, binding, and pore formation; and are associated with chemotaxis properties (White et al. 1995; Wu et al. 2003; Klüver et al. 2006). Changes in disulfide bonding numbers and topology can influence the three dimensional structure of defensins, affecting their antimicrobial potency, chemotaxis, and the efficiency of CCR6 and monocyte binding (Mangoni et al. 1996; Wu et al. 2003; Klüver et al. 2005; Varkey and Nagaraj 2005). However, alterations to the 6-cysteine motif can also be beneficial and increase the antimicrobial repertoire, as observed in rat and mice with defensin variants containing 5, 9, or 11 cysteines (Campopiano et al. 2004; Andersson et al. 2012; Patil et al. 2013).

Hydrophobicity is an important factor governing antimicrobial potency of peptides (Klüver et al. 2005; Klüver et al. 2006). The alpha and beta defensins we identified have a high proportion of hydrophobic residues >30% (Figs. 1 and 2; Supplementary Table 4). Hydrophobic residues cluster in the signal peptide of both subfamilies, which facilitates defensin transmembrane transport. Hydrophobic residues are commonly located in close proximity to positively charged amino acids, which is a noted structural feature of cytolytic peptides (Kini and Evans 1989) including previously characterized defensins (Yang et al. 2000).

Due to proportionally high arginine and lysine content, 112 of the defensins identified were positively charged (Supplementary Table 4). Beta defensins had a charge ranging from −5.4 to 15.4 (average 4.6), while alpha defensins were all cationic with charge ranging from 2.7 to 10.7. Peptide charge plays a crucial role in antimicrobial activities and immunomodulatory functions of defensins (Klüver et al. 2005). We identified eight beta defensin mature peptides with negative or neutral charges (Supplementary Table 4). While this is not widely reported for defensins (Yenugu et al. 2004; Wei et al. 2015),

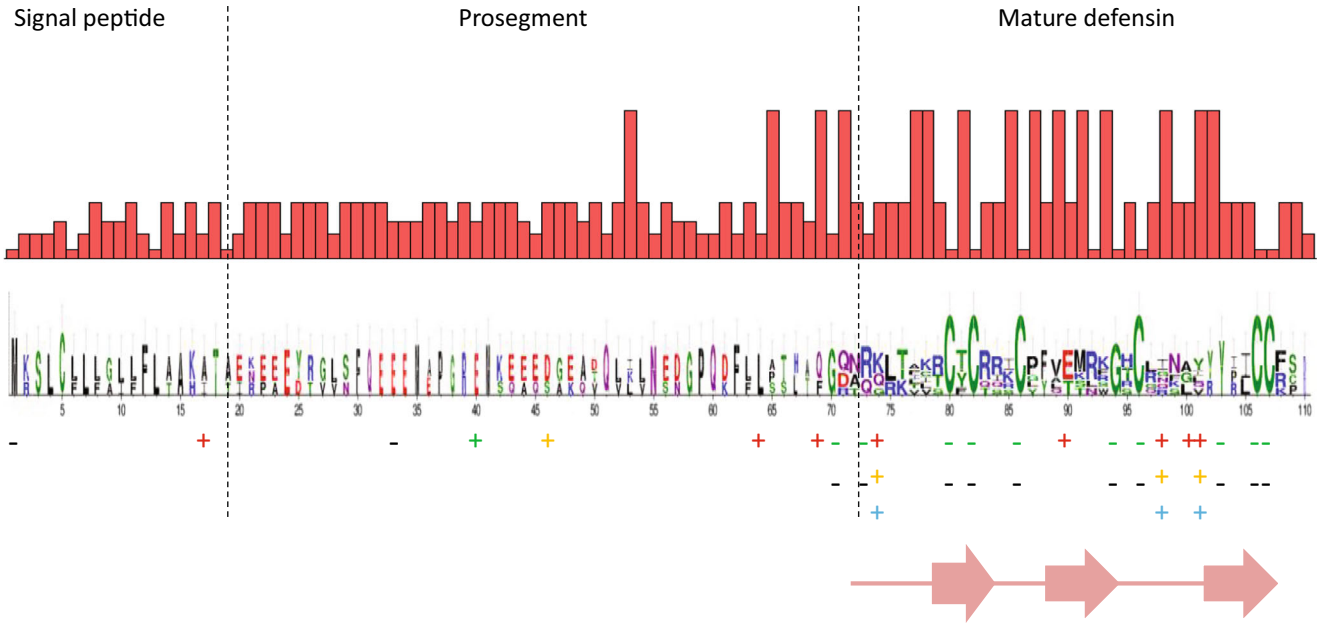

Fig. 1 A peptide sequence logo for marsupial alpha defensins showing full-length gene variability and amino acid sites under selection ($p < 0.05$) according to FEL, MEME, and FUBAR tests. Negatively selected sites shown by a dash (−) (FEL green, FUBAR black) and positively selected site shown by a plus (+) (FEL *blue*, FUBAR *yellow*, MEME *red*). The positions of the β-strands within the mature peptide are shown beneath the logo. Basic residues are *blue*, acidic residues are *red*, hydrophobic residues are *black*, polar residues are *green*, and neutral residues are purple

anionic antimicrobial peptides such as dermcidin from the human (Steffen et al. 2006) and maximin H5 from the toad *Bombina maxima* (Lai et al. 2002) possess innate immune functions and antimicrobial properties, indicating charge alone does not fully account for antimicrobial potential. Alpha defensins characteristically have a long anionic propiece, which acts to balance the cationic charge in the mature peptide domain to reduce autocytotoxicity prior to secretion (Michaelson et al. 1992; Hughes and Yeager 1997). The classical 6-cysteine alpha defensins that we annotated had a mature peptide charged between 9.7 and 10.7 (mean 10.1) and an anionic signal/prosegment peptide between −8.1 and −13.8 (mean −11.0), which conform to the charge balance theory. However, this charge balance was not observed for the 8-cysteine alpha variants, which had prosegments with a slightly negative charge ranging from −0.1 to −0.9.

One hundred and twelve defensins identified in this study have the characteristic hallmarks of cationic antimicrobial defensins. Cationic antimicrobial peptides are being actively investigated as source for novel antibiotics (Aoki and Ueda

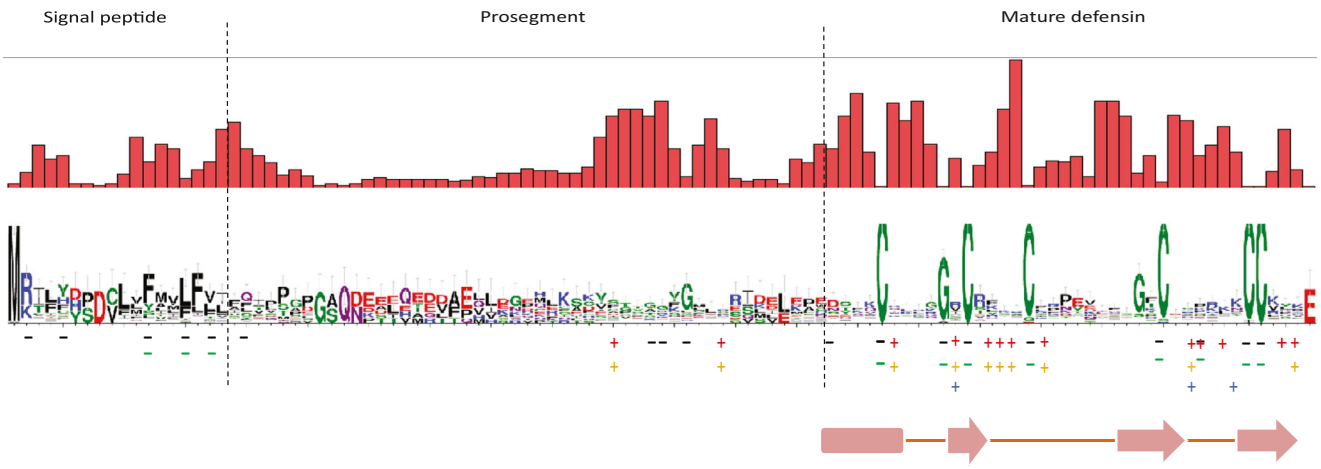

Fig. 2 A peptide sequence logo for marsupial beta defensins showing full-length gene variability and amino acid sites under selection ($p < 0.05$) according to FEL, MEME, and FUBAR tests. Negatively selected sites shown by a dash (−) (FEL *green*, FUBAR b*lack*) and positively selected site shown by a plus (+) (FEL *blue*, FUBAR *yellow*, MEME *red*). The positions of the α-helix, β-strands, signal/prosegment and mature peptide are shown beneath the logo. Basic residues are *blue*, acidic residues are *red*, hydrophobic residues are *black* and neutral residues are *purple*

2013). Mimetics are currently in clinical trial stages and show promising results against skin infections and *Staphylococcus aureus*, as well as broad spectrum in vitro activity against multidrug-resistant gram positive and negative bacteria (Tew et al. 2009; Mensa et al. 2014). The unique marsupial cysteine variants identified, and those defensins with high charge and hydrophobic properties, may be good targets for novel therapeutic development.

Synteny analysis and phylogenetics

Vertebrate defensins are arranged in chromosomal clusters that each span less than 1.2 Mb (Patil et al. 2004; Patil et al. 2005). Human, mouse, and opossum have 39, 52, and 37 beta defensin genes in three, four, and five clusters, respectively (Patil et al. 2004; Patil et al. 2005) (Fig. 3). The Tasmanian devil, koala, and wallaby have a similar number of beta defensins (48, 34, and 39). The Tasmanian devil defensins are located on 13 supercontigs that map to chromosome 1 and chromosome 4. Koala genes are yet to be mapped to chromosmes, but due to the high quality of the genome assembly, all defensins except DefB25 can be located on three scaffolds.

Tasmanian devil and koala defensins belong to three syntenic clusters (cluster A, C, D) as in the opossum (Fig. 3). These clusters share synteny with eutherian gene clusters A, C, and D (Fig. 3). Beta defensin expansions have

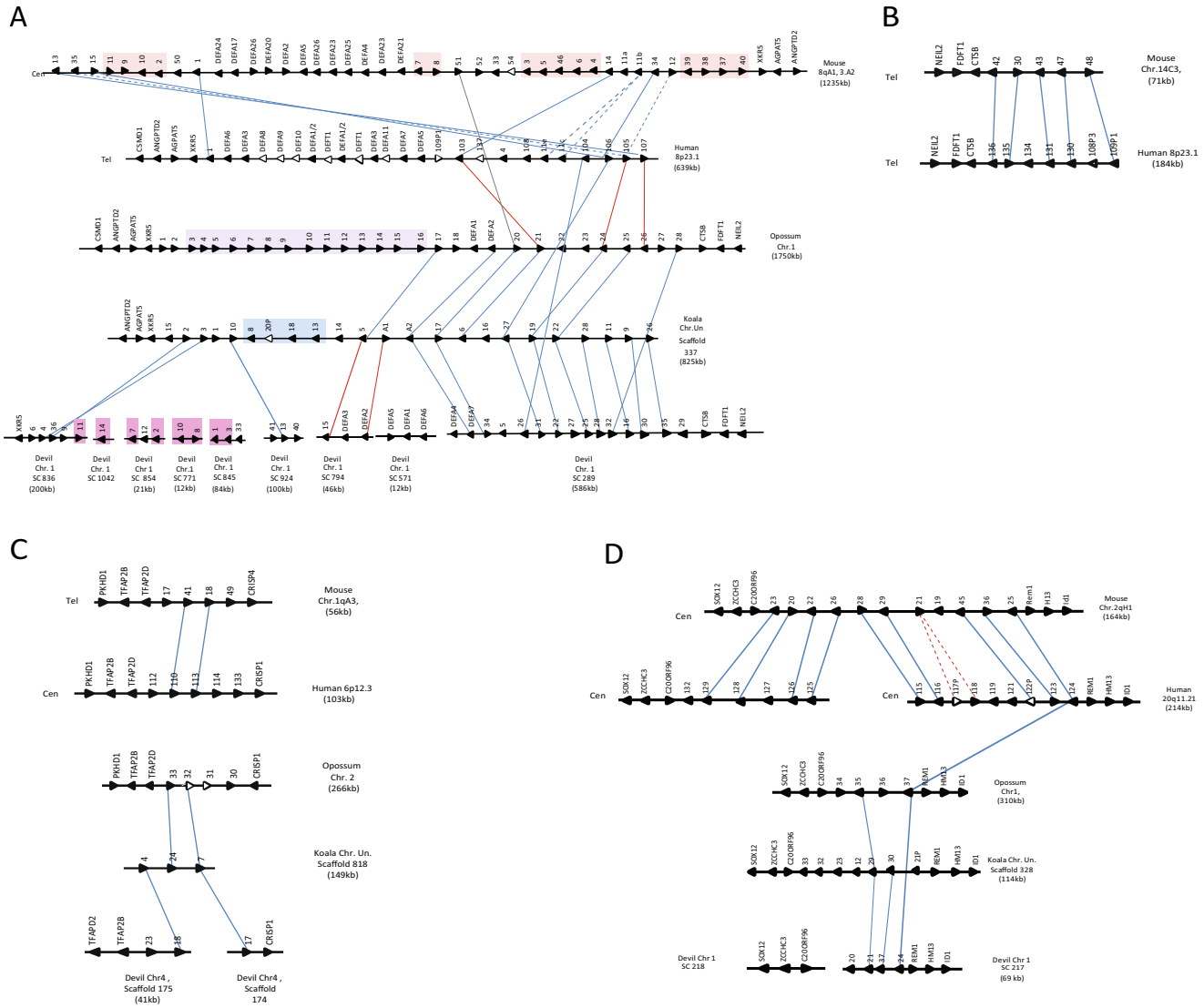

Fig. 3 Genomic organization of alpha and beta defensins in eutherians and marsupials. The position and orientation of genes are represented by triangles. Genes with labels containing solely numbers are beta-defensins. *Solid lines* link orthologous genes across species and *dotted lines* represent paralogs. Orthologs and paralogs were determined based on the phylogenetic analysis where *red lines* indicate bootstrap support between >60 and 79% and *blue lines* indicate bootstrap value >80%. Size indicated under chromosome number is the defensin cluster size excluding flanking genes. *White filled arrows* indicate pseudogenes; *colored boxes* around genes indicate species-specific duplications which are also highlighted in the phylogenetic trees

occurred in the Tasmanian devil, koala, tammar wallaby opossum, and mouse lineages (Fig. 4). Genes within these expansions have no clear orthologs, and in the koala, Tasmanian devil, and mouse, they tend to cluster together in the genome. This suggests that the expansions are the result of recent gene duplications and, as has been proposed in mice, are likely an adaptive response to species-specific pathogenic challenges (Patil et al. 2004). It has been proposed that antimicrobial peptide gene expansions in marsupials are associated with their reproductive physiology (Wang et al. 2011; Peel et al. 2016). Marsupials give birth to immunologically naïve young that develop in a non-sterile pouch. It is possible that defensin duplications have evolved in marsupials to protect the immunologically naïve young as broad-spectrum natural antibiotics (Belov et al. 2007). As mice also have short gestation periods and are born with less mature immune systems compared to

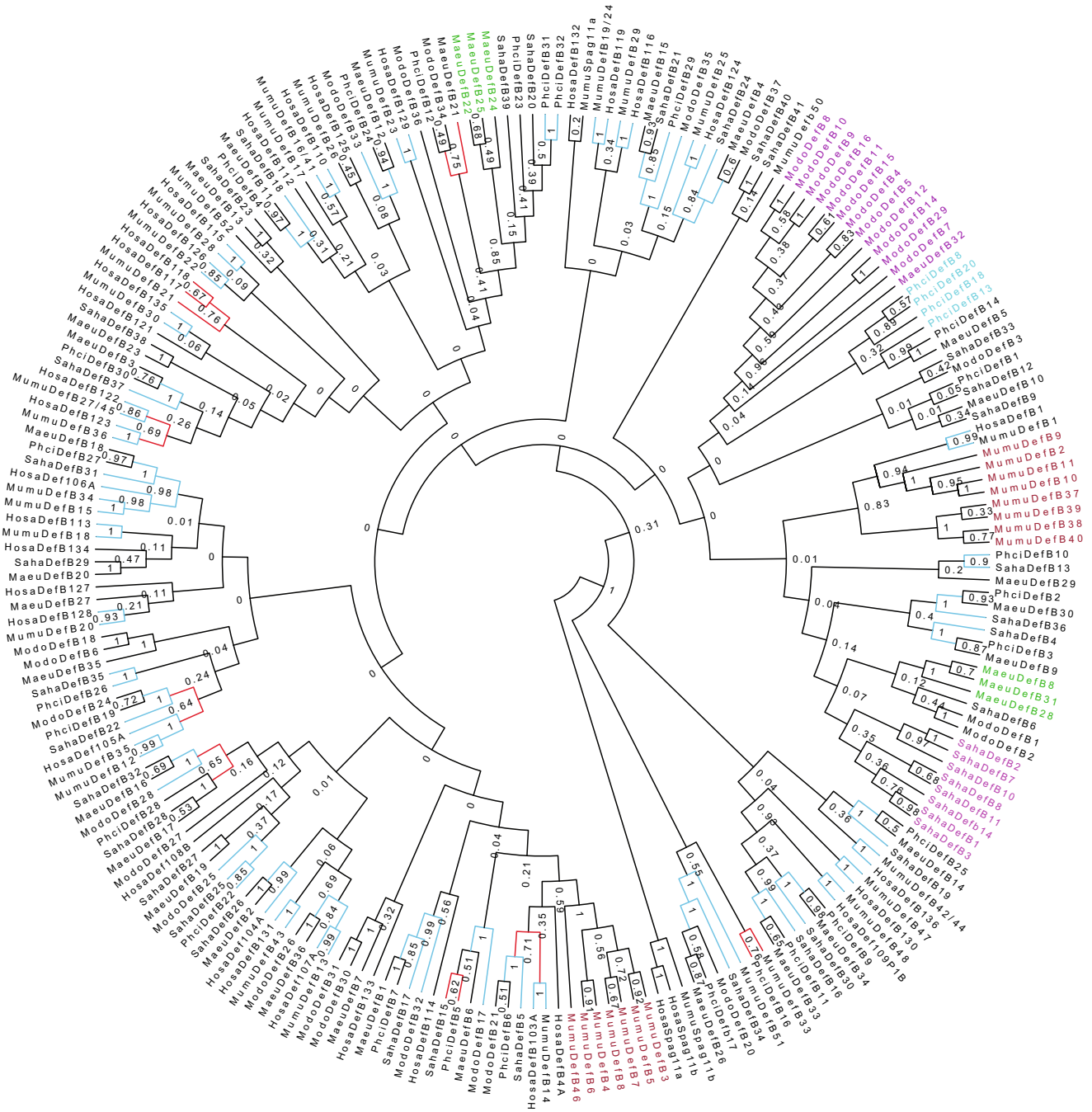

Fig. 4 Phylogenetic tree showing relationship between human, mouse, opossum, Tasmanian devil, koala, and tammar wallaby beta defensins. Bootstrap values are shown next to the nodes; nodes with bootstrap support of 60–79 and >80% are highlighted in *red* and *blue*, respectively. Species-specific expansions are highlighted: mouse (*maroon*), koala (*blue*), devil (*pink*), tammar wallaby (*green*), opossum (*purple*)

humans (Chappuis 1998), mouse beta defensin duplications may also be associated with reproductive traits.

Beta defensin gene expansions in the Tasmanian devil, koala, opossum, and mouse are all located within the largest syntenic cluster A (Fig. 3), which also contains the alpha defensin family in all species. Alpha defensins are thought to share a common ancestral beta defensin (Liu et al. 1997; Patil et al. 2004). In contrast to beta defensins, there are no clear orthologs between eutherian and marsupial alpha defensins (Fig. 5). Alpha defensins in mice and rats have evolved in a lineage-specific manner, whereas orthologs are shared between humans and primates, and within the marsupials (Fig. 5).

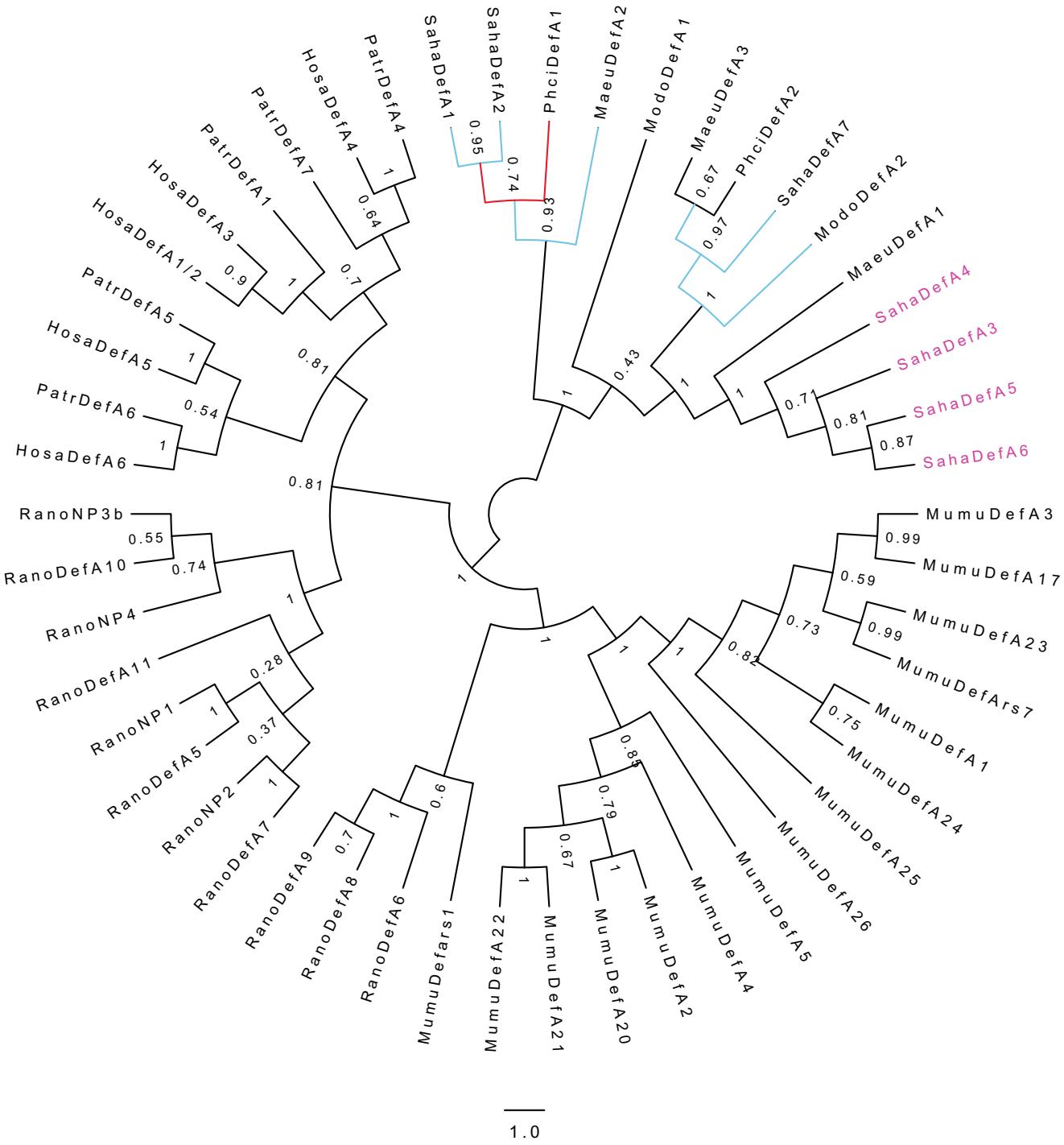

Fig. 5 Phylogenetic tree showing relationship between human, mouse, opossum, Tasmanian devil, koala, and tammar wallaby alpha defensins. Bootstrap values are shown next to the nodes; nodes with bootstrap support of 60–79 and >80% are highlighted in *red* and *blue*, respectively. The Tasmanian devil-specific expansions are highlighted in pink

Tasmanian devils are the only marsupial to have an expansion in the alpha defensin family, possessing seven alpha defensins compared to other marsupials that have two. Three devil alpha defensins were transcribed in the facial tumor and one was transcribed in the testis. As alpha defensin expression is generally limited to myeloid cells, intestinal paneth cells, and reproductive epithelial cells (Selsted and Ouellette 2005), further characterization of the expression and function of these genes will help determine the significance of this expansion in the devil. Considering the carrion-scavenging diet of devils (Pemberton et al. 2008), a heightened defense against foodborne pathogens may be a possible explanation to this devil-specific alpha defensin expansion.

The conserved synteny of defensin gene regions across species is supported by the order of genes that flank the clusters. Interestingly, the flanking genes *CTSB*, *FDFT1*, and *NEIL2* of Tasmanian devil and opossum cluster A are found downstream of mouse and human cluster B defensins (Fig. 3). This suggests that the mouse and human cluster B defensins were likely translocated from cluster A to a different chromosomal region during eutherian evolution. No clear orthologous relationship between eutherians and marsupials was inferred in cluster B and C (Fig. 3), indicating that cluster B and C defensins may have evolved independently in each lineage after the marsupial eutherian divergence.

Natural selection on marsupial defensin genes

A comparison of synonymous (d_S) and nonsynonymous (d_N) substitution rates revealed that an overall purifying selection ($d_N < d_S$ significance 0.05) was acting across both exons of marsupial beta defensins and across the second exon of alpha defensins (Table 1).

Thirteen codon sites in alpha defensins (Fig. 1) and 18 sites in beta defensins (Fig. 2) were under purifying selection. The majority of negatively selected sites in both subfamilies was concentrated in the mature peptide domain and involved mainly polar residues, including the six conserved cysteines. In the alpha defensin signal peptide, five sites were under purifying selection, most of which are hydrophobic. Negative selection of these polar and hydrophobic sites is likely due to the essential function of these residues, which are required to maintain defensin structural integrity, transport, and the biological functions discussed earlier.

Overall, there were nine sites under positive selection in marsupial alpha defensins and 16 sites in beta defensins. Like the distribution of negatively selected sites, positively selected sites were also concentrated in the mature peptide in both subfamilies, with five sites under positive selection in the alpha mature peptide and 11 in the beta mature peptide. These sites mostly contain hydrophobic or charged residues and are located mainly within the loop regions between the antiparallel beta sheets, or within N-terminal or C-terminal positively charged clusters. The defensin loops and terminal charge clusters have previously been shown to protrude from the defensin core and have functions associated with membrane docking and microbial membrane integration (Romestand et al. 2003). The close proximity of sites under negative and positive selection in the mature peptide suggests coordinated selection acts to maintain this variable loop region. Positive selection favoring charge alterations has been previously shown to be a driver of mammalian defensin gene divergence (Hughes 1999; Semple et al. 2003). This charge-favoring selection is a hallmark of the evolutionary arm race between cationic defensins and their anionic microbial targets (Semple et al. 2003).

Conclusion

In this study, we characterized defensin genes of the Tasmanian devil, koala, and tammar wallaby and performed the first evolutionary comparison of defensins across multiple marsupial species. This work provides an important first step in characterizing the function of these peptides in marsupial immunity.

AMP, antimicrobial peptide; DefA, alpha defensin; DefB, beta defensin; HMM, hidden Markov model; Hosa, human; Maeu, tammar wallaby; Modo, opossum; Mumu, mouse; Phci, koala; Saha, Tasmanian devil.

Table 1 Mean rates of synonymous (d_S +/− S.E) and nonsynonymous (d_N +/− S.E) nucleotide substitutions and test for overall selection in alpha and beta defensins

Defensin subfamily	Signal peptide and propiece				Mature peptide			
	d_S	d_N	Stat[a]	P[a]	d_S	d_N	Stat[a]	P[a]
Beta defensins	1.005 ± 0.074	0.585 ± 0.088	3.934	<0.001	1.335 ± 0.058	0.966 ± 0.136	2.096	0.019
Alpha defensins	0.643 ± 0.085	0.508 ± 0.062	1.533	0.064	0.800 ± 0.185	0.395 ± 0.180	2.066	0.020

[a] Z test statistics for purifying selection ($d_S > d_N$)

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Authors' contributions KB, YC, and DOM conceived and designed the study. EAJ carried out analyses under the supervision of YC and DOM and drafted the paper. KB, YC, and DOM revised the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethics approval No ethics approval was required for this study.

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