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Evolution of caprine and ovine β-defensin genes

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Abstract Defensins comprise an important family of anti-microbial peptides. Among vertebrates, numerous defensin genes have been detected, but their evolutionary background is still discussed. We investigated the molecular evolution and variability of β-defensins of Caprini via sequence analyses of defensin introns. Screening of several domestic and wild species of Caprini revealed a total of 13 discrete β-defensin coding sequences, with three of them described before this study. Phylogenetic analyses revealed that the array of newly described defensin genes is of monophyletic origin and has arisen in numerous independent duplication events after separation of the ancestral defensins. As a result of that scenario, recent defensin genes are distributed in a species-specific manner. Values of synonymous and non-synonymous substitutions demonstrated that both modes of evolutionary pressure, positive as well as negative selection, have acted. In addition, conservation of some β-defensin exons is demonstrated. Discrimination of certain β-defensin genes was possible only due to intron-specific differences. Therefore, sequence analyses restricted to the exons would result in underestimation of the number of β-defensin genes. Our study shows that for reconstruction of the phylogenetic history data of defensin introns are more appropriated. Comparisons among the amino acid sequences show moderate substitutions without changing the net charge of the mature peptides.

Keywords Immune defence · Innate immunity · Molecular evolution · *Capra* · *Ovis*

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Introduction

Cationic anti-microbial peptides are multifunctional peptides of the innate immune system which act directly against host-invading micro-organisms (Boman 1991; Hancock and Lehrer 1998; Lehrer et al. 1993; Martin et al. 1995; Nicholas and Mor 1995). In addition, they trigger the specific immune response via signalling between the early and the specific immune response (Hoover et al. 2002; Yang et al. 1999, 2004). The anti-microbial execution is thought to be caused by the attachment of the cationic peptides (positively charged) at the anionic phospholipids of the microbe's membrane, resulting in its disruption and cell death (Ganz 2003; Kagan et al. 1990; Satchell et al. 2003). One of the most prominent components of that array of anti-microbial peptides is represented by defensins. Defensins are small (2–6 kDa), cationic and contain six cysteine residues at defined positions. Based on their size and the spatial position of the six cysteine residues, defensins of higher vertebrates are classified as α-, β- and θ-defensins. Defensins are translated as unmature precursors and are encoded by two separate exons, of which one encodes the signal sequence and the other the pro- and the mature peptide (reviewed in Ganz 2003; Hughes 1999; Lehrer and Ganz 2002; Schröder 1999; Schutte and McCray 2002; Wang et al. 2003).

The modern era of anti-microbial peptide research began with the description of lysosomal cationic peptides in leucocytes from rabbit and guinea pig (Zeya and Spitznagel 1966), later characterised structurally and classified as α-defensins (Selsted et al. 1985). Up to date α-defensins were also found to be present in human (Gabay et al. 1989; Ganz et al. 1985; Jones and Bevins 1992, 1993; Patil et al. 2004;

Territo et al. 1989; Wilde et al. 1989) and non-human primates (Patil et al. 2004; Tanabe et al. 2004; Tang et al. 1999a), mice (Ouellette et al. 1992, 1994, Patil et al. 2004), hamsters and rats (Patil et al. 2004) (reviewed in Raj and Dentino 2002). Because no α-defensins from outside the mammals were detected so far, they seem to be specific to mammals.

β-Defensins were also detected in numerous mammalian species, e.g. primates (Boniotto et al. 2003a,b; Del Pero et al. 2002), human (Bensch et al. 1995; Schutte et al. 2002), cattle (Diamond et al. 1991; Selsted et al. 1993), goat (Zhao et al. 1999), sheep (Huttner et al. 1998) and mice (Bals et al. 1998; Huttner et al. 1997; Jia et al. 2000; Schutte et al. 2002), as well as in birds (Brockus et al. 1998; Harwig et al. 1994), where they are called gallinacins.

θ-Defensins are cyclic defensins that have been identified so far only in primates (Nguyen et al. 2003; Tang et al. 1999b). They are generated by post-translational ligation of two truncated α-defensins (Tang et al. 1999b).

α- and β-defensins seem to be distinct regarding their tissues of expression; α-defensins are mainly expressed by leucocytes and Paneth cells of the small intestine (Ganz 2003; Lehrer and Ganz 2002; Ouellette 2004; Schutte and McCray 2002), vertebrate β-defensins are predominantly produced by epithelial cells lining various organs, e.g. the human skin and bronchial tree, the tongue and the genito-urinary tract (Diamond et al. 1991; Diamond and Bevins 1998; Harder et al. 1997; Schonwetter et al. 1995).

Several studies were carried out to examine the evolutionary relationships of α- and β-defensins detected in several mammalian species (Hughes 1999; Hughes and Yeager 1997; Liu et al. 1997; Morrison et al. 2003). Based on the complete genome sequences of mouse, human and chicken, some studies applied a computational search for defensin coding sequences (Patil et al. 2004; Schutte et al. 2002; Semple et al. 2003; Xiao et al. 2004). In conclusion, the defensin genes were found to be arranged in clusters (Maxwell et al. 2003; Morrison et al. 2003; Patil et al. 2004; Schutte et al. 2002; Semple et al. 2003; Xiao et al. 2004), characterised by a high sequence divergence of discrete genes. This data further indicated rapid duplication and diversifying positive selection of both α- and β-defensins. In addition, the physical linkage of α- and β-defensin clusters in the genome of mouse and human was detected (Liu et al. 1997; Schutte et al. 2002), which may suggest a common ancestry of α- and β-defensins.

However, little is known about the repertoire and evolutionary relatedness of β-defensins belonging to mammalian species outside from the sequenced ones. Studies with numerous mammalian defensins suggested several duplication events following positive selection and diversification within each group studied (Antcheva et al. 2004; Hughes 1999; Hughes and Yeager 1997; Maxwell et al. 2003; Morrison et al. 2003; Semple et al. 2003). In a similar approach, Luenser and Ludwig (2005) estimated the sequence divergence and molecular evolution of bovine β-defensins and found evidence for a common ancestry of different subfamilies of β-defensins as well as lineage-specific evolution. However, all these studies have in common is to be solely based on the defensin's exons, which in several cases were shown to evolve under positive selection. Positively selected genes are characterised by an accelerated evolution likely rendering the development of similar features. Thus, detection of sequence similarity may be explained by a common ancestry as well as by a process of convergent adaptation. It is therefore useful for evolutionary investigations to compare sequences evolving under a neutral mode of evolution. For β-defensins, such sequence is the intron separating exon 1 and exon 2 (Fig. 1). Here we present an array of newly detected β-defensins of members of the tribe Caprini, obtained via a PCR-based genomic search, and explain their evolutionary history, accentuating their importance for the vertebrate immune defence.

Materials and methods

The following species of the genera *Ammotragus*, *Pseudois*, *Capra* and *Ovis*, all belonging to the tribe Caprini, were used for molecular investigation: *Capra hircus* (domestic goat), *Ovis aries* (domestic sheep), *Ovis ammon* (argali sheep), *Pseudois nayaur* (blue sheep), *Ammotragus lervia* (barbary sheep) and *Ovis orientalis* (urials and mou-

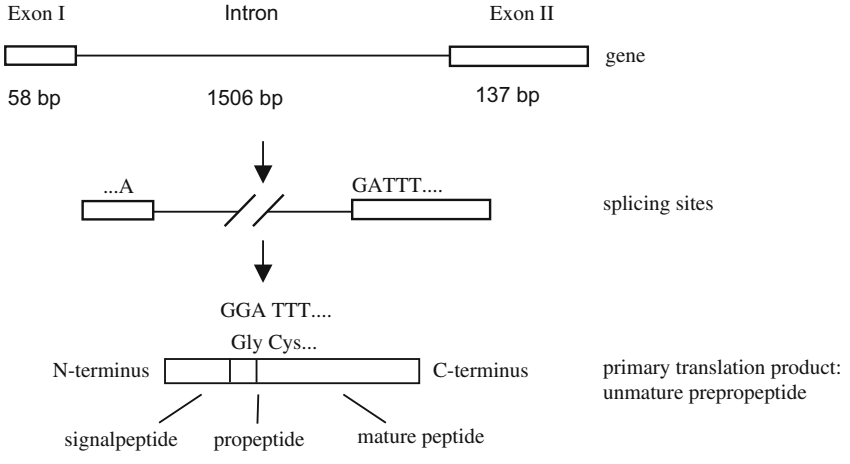

Fig. 1 Genetic structure of a β-defensin gene. Lengths of both exons and of the intron as well as the splicing sites of the mRNA are shown. The primary translation product is an unmature precursor consisting of a signalpeptide at the N-terminus, a mature peptide at the C-terminus and an intervening propiece

flons), represented by two subspecies: *O. orientalis severtzovi* (Severtzov's urial) and *O. orientalis cycloceros* (Afghan urial). Nomenclature and common names are based on the geographic origin of samples following the recommendations of the International Union for Conservation of Nature and Natural Resources (IUCN) Wild Sheep and Goat Specialist Group (Shackleton 1997). Total DNA was purified from blood and tissue samples according to standard protocols (QIAGEN Inc., The Netherlands). For amplification of the complete defensin genes, we designed the primers P1 5′-ATCAGCTGCAGAGCTCGT GA-3′ and P2 5′-CAACCTCAATGACCAGTGG-3′. The forward primer (P1) annealed 27 bp upstream of the first exon, and the reverse primer (P2) annealed 132 bp downstream of the second exon. Amplification was performed using standard conditions, with annealing at 58°C. PCR products, 1,860 bp in length, were cloned with the TA-TOPO cloning kit (Invitrogen, The Netherlands). The

Table 1 Multiple nucleotide alignment of caprine defensins

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                                 1111111111222222222233333333334444444444555555555566666666
                        12345678901234567890123456789012345678901234567890123456789012345678
sbd2 (U75251)           ATGAGGCTCCATCACCTGCTCCTCGTGCTCTTCTTCGTGGTCCTGTCTGCTGGGTCAGGATTTACT
sbd2_1                  ............................................................
sbd2_2                  ............................................................
sbd2_3                  ............................................................
sbd2_4                  ............................................................
sbd2_5                  ............................................................
sbd1 (U75250)           ............................................................
sbd1_1                  ............................................................

chbd1 (Y17679)          ATGAGGCTCCATCACCTGCTCCTCGTGCTCTTCTTCCTGGTCCTGTCTGCTGGGTCAGGATTTACT
chbd1_1                 ..................................G.........................
chbd2 (AJ009877)        ......................C.....................................
chbd2_1                 ......................C.....................................
pn_1                    ..................................G.........................
pn_2                    ..................................G.........................
pn_3                    ..................................G.........................


                                                          1111111111111111111111111111111
                        66677777777778888888888999999999900000000001111111112222222222233
                        78901234567890123456789012345678901234567890123456789012345678901

sbd2 (U75251)           CATGGAGTAACAGATAGTCTAAGCTGCCGTTGGAAGAAAGGCATCTGTGTGCTGACCAGGTGCCC
sbd2_1                  ............................................................
sbd2_2                  ............C...........A...................................
sbd2_3                  ..A.........C...........A...................................
sbd2_4                  ............C...........A...................................
sbd2_5                  ............................................................A.....
sbd1 (U75250)           ..A.......G.A..C...........A.A....T......G.........C..G.........
sbd1_1                  ..A.......G.A..C...........A.A....T......G.........C...........

chbd1 (Y17679)          CAAGGAATAAGAAGTCGTCGAAGCTGCCATAGGAATAAAGGCGTCTGTGCGCTGACCAGGTGCCC
chbd1_1                 ............................................................
chbd2 (AJ009877)        ..........T..A..A..........T.....................C.G.........
chbd2_1                 ..........T..A..A.................T.................C.GA.......
pn_1                    ......G......A.................................T.............
pn_2                    ......G......A.................................T.............
pn_3                    ......G......A..................G..............T.............


                        1111111111111111111111111111111111111111111111111111111111111111
                        3333333344444444445555555556666666666777777777788888888889999999
                        2345678901234567890123456789012345678901234567890123456789012345

sbd2 (U75251)           TGGAACCATGAGACAGATTGGCACCTGTTTCGGGCCCCCAGTAAAATGCTGCAGACTGAAGTAA
sbd2_1                  .A..........................................................
sbd2_2                  ..........................................................GAA.....
sbd2_3                  ..........................................................GAA.....
sbd2_4                  ............................................................
sbd2_5                  ............................................................
sbd1 (U75250)           .A..CA.....................CG........................AA.......
sbd1_1                  .A..CA.....................CA........................AA.......

chbd1(Y17679)           TAGAAACATGAGACAGATTGGCACCTGTTTCGGGCCCCCAGTAAAATGCTGCAGAAAGAAGTAA
chbd1_1                 ............................................................
chbd2(AJ009877)         ..........................CA................................
chbd2_1                 ............................................................
pn_1                    .....C.....................CA........A.......................
pn_2                    .....C.....................CAT.......................CT......G
pn_3                    .....C.....................CA........A.......................
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Vertical numbers indicate nucleotide positions. Dots indicate identical sites referring to *sbd*2 and *chbd*1. Intron sequences are excluded

cloned PCR products were sequenced with several internal primers. Sequencing was carried out on an automated ABI 3100 DNA sequencer (Applied Biosystem, USA). Coding sequences composed of exons 1 and 2 were aligned with published β-defensin genes retrieved from GenBank (AJ00 9877, Y17679, U75250, U75251) using molecular evolutionary genetics analysis (MEGA) 2.0 (Kumar et al. 1993). The assignment of the ovine and caprine β-defensins obtained in this study to the different defensins was performed using nucleotide Basic Local Alignment Search Tool (BLAST) search in the National Center for Biotechnology Information (NCBI) GenBank. The phylogenetic trees were constructed by neighbor-joining (NJ) algorithms implemented in phylogenetic analysis using parsimony (*and other methods) (PAUP*) 4.0b10 (Swofford 2002) and MEGA 2.0 (Kumar et al. 1993) based on the proportion of nucleotide sites at which the sequences compared were different. Their reliability was assessed by 1,000 bootstrap replications. The significance of branch lengths in the NJ tree was also examined by a standard error test using the confidence probability (CP) program of MEGA. To test if sequences followed the neutral model of molecular evolution (Kimura 1969), the following tests, implemented in the software package DnaSP (v. 4.0; Rozas et al. 2003), were carried out: Hudson–Kreitman–Aguade (HKA) test (Hudson et al. 1987), Tajima's *D* test (Tajima 1989), Fu and Li's *D** test (Fu and Li 1993) and McDonald–Kreitman test (McDonald and Kreitman 1991). The model of evolution was determined using MODEL TEST 3.6 (Posada and Crandall 1998). Calculation of the number of synonymous (dS) substitutions per synonymous site and non-synonymous (dN) substitutions per non-synonymous site was done by the method of Nei and Gojobori (1986), likewise using the program DnaSP4.0 (Rozas et al. 2003).

Results

Genomic DNA of specimens belonging to the tribe Caprini, representing two domestic species (*O. aries* and *C. hircus*) and four related wild species (*O. ammon*, *P. nayaur*, *A. lervia* and *O. orientalis*, represented by the two subspecies, *O. orientalis cycloceros* and *O. orientalis severtzovi*), was screened for β-defensin genes. Defensin genes of Caprini are composed of two exons with 58 bp (exon 1) and 137 bp (exon 2), respectively, which enclose a single intron of 1,506 bp (Fig. 1). Sequences of exons 1 and 2 are reported in Table 1.

A total of 13 discrete β-defensin coding sequences was detected (Table 2), including two sequences already known from *O. aries*: sheep β-defensin 1 [*sbd*1 (U75250)] and sheep β-defensin 2 [*sbd*2 (U75251)]. Interestingly, in this study, *sbd*1 was detected in the related wild sheep, *O. orientalis cycloceros*. Newly detected sequences identified as variants of previously published defensins from *C. hircus* (Y17679 and AJ009877) and *O. aries* (U75250 and U75251) were numbered consecutively (Table 2). With the exception of defensin genes from the wild and the highly endangered species *P. nayaur*, all defensin genes can be designated as variants of either *C. hircus* β-defensins 1 [*chbd*1 (Y17679)] and 2 [*chbd*2 (AJ009877)], or sheep β-defensins 1 [*sbd*1 (U75250)] and 2 [*sbd*2 (U75251)].

Comparison of all variants demonstrated high substitution rates, both inter- and intraspecifically. To understand these substitution patterns, we analysed the degree of phylogenetic relatedness of the detected caprine and ovine β-defensins. Concerning our aim to reconstruct the evolutionary history of newly identified defensins, we regarded the non-coding intron sequences to be most suitable for such analysis.

Table 2 Distribution of newly detected β-defensin genes within four taxa of Caprini (*Pseudois*, *Ammotragus*, *Capra* and *Ovis*)

Published similar sequence	This study	Species	Positions of diagnostic substitutions	Consequence of substitutions
*sbd*1 (U75250) *O. aries*	*sbd*1	*O. orientalis cycloceros*	–	
	*sbd*1_1	*O. aries*, *A. lervia*	122, 161	$S^{41}\rightarrow T^{41}$, $R^{54}\rightarrow H^{54}$
*sbd*2 (U75251) *O. aries*	*sbd*2	*O. aries*	–	
	*sbd*2_1	*O. orientalis cycloceros*	133	$G^{45}\rightarrow R^{45}$
	*sbd*2_2	*O. orientalis severtzovi*, *O. ammon*	82, 95, 186, 187, 188	$S^{28}\rightarrow R^{28}$, $R^{32}\rightarrow H^{32}$, $L^{63}\rightarrow K^{63}$
	*sbd*2_3	*O. orientalis severtzovi*	69, 82, 95, 186, 187, 188	$H^{23}\rightarrow Q^{23}$, $S^{28}\rightarrow R^{28}$, $R^{32}\rightarrow H^{32}$, $L^{63}\rightarrow K^{63}$
	*sbd*2_4	*O. ammon*	82, 95	$S^{28}\rightarrow R^{28}$, $R^{32}\rightarrow H^{32}$
	*sbd*2_5	*O. ammon*	125	$R^{42}\rightarrow K^{42}$
*chbd*2 (AJ009877)	–			
C. hircus	*chbd*2_1	*C. hircus*	94, 101, 122, 160, 161	$Y^{32}\rightarrow H^{32}$, $N^{34}\rightarrow I^{34}$, $A^{41}\rightarrow D^{41}$, $H^{54}\rightarrow F^{54}$
*chbd*1 (Y17679)	–			
C. hircus	*chbd*1_1	*C. hircus*	37	$L^{13}\rightarrow V^{13}$
	*pn*_1	*P. nayaur*		
	*pn*_2	*P. nayaur*		
	*pn*_3	*P. nayaur*		

For better comparison, hosts of four highly similar defensins (*chbd*1, *chbd*2, *sbd*1 and *sbd*2) detected before this study are given

The NJ tree, based on the intron sequences of ovine and caprine defensins (Fig. 2), shows that β-defensins of the tribe Caprini form four distinct species-specific lineages: (1) ovine defensins harbouring all detected defensins of *Ovis*, (2) caprine defensins harbouring defensins of *Capra*, (3) defensins of *Pseudois* and (4) defensins of *Ammotragus*. Obviously, β-defensins of *Ovis*, *Capra*, *Pseudois* and *Ammotragus* are homologues derived from a common ancestor. Species-specific clustering indicates lineage-specific evolution. Interestingly, defensins of *Capra* and *Pseudois* form a monophyletic group, indicating a closer relation between defensins from *Pseudois* and *Capra* than between those of *Pseudois* and *Ovis*. Separation of these clusters is supported by high bootstrap values (Fig. 2).

The defensin gene tree, based on the exon regions (Fig. 3), shows that sequences of *Pseudois*, *Ammotragus* and *Capra* grouped together separated from the wild sheep defensins. Thus, the exon tree contradicts the presented intron tree.

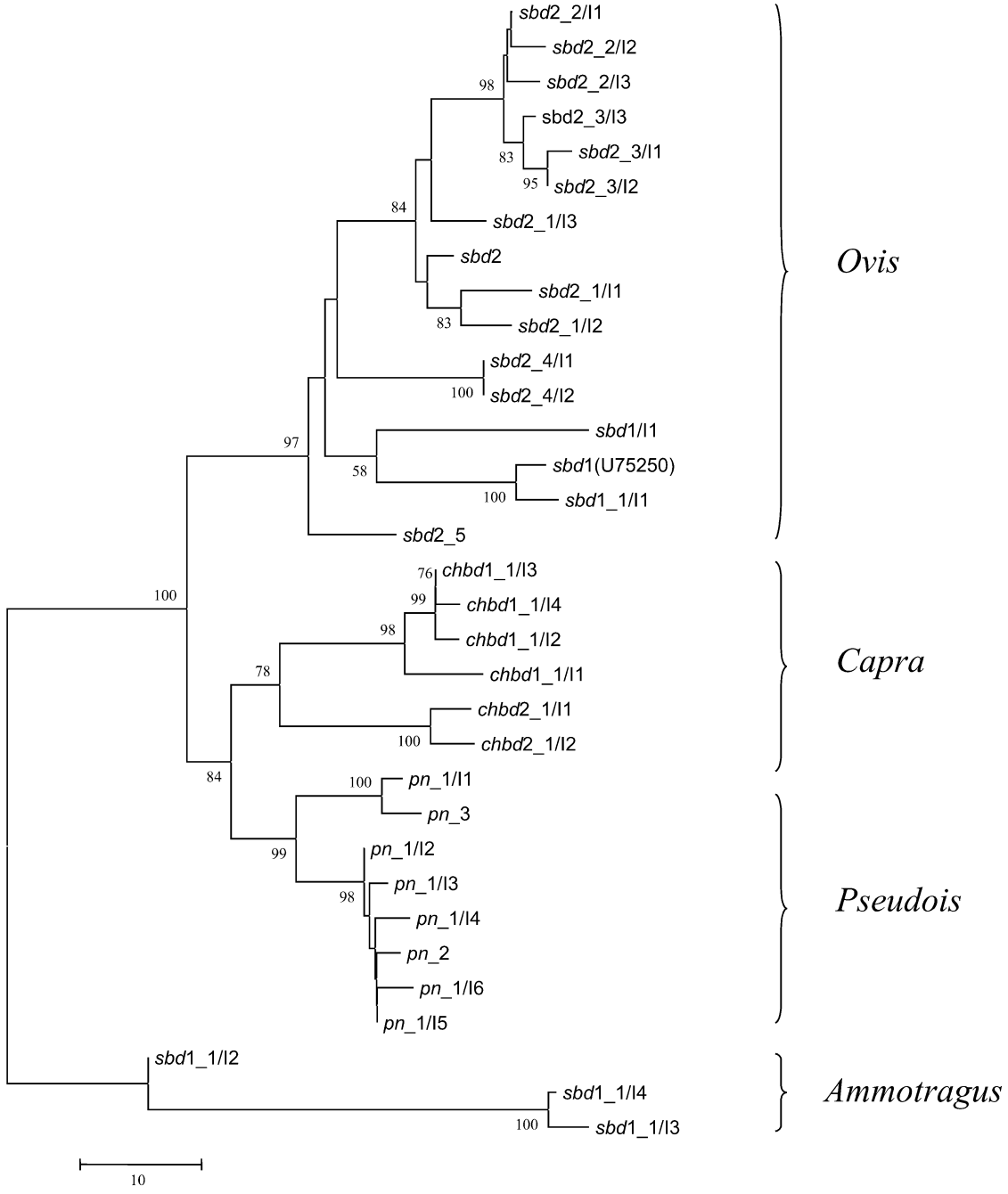

Fig. 2 NJ tree based on the proportion difference of nucleotides, using exclusively intron sequence information. Sequences identical in coding regions but differing in introns are termed as intron variants of the corresponding gene (*sbd*2/I1, *sbd*2/I2 etc.). The reliability of each branch was tested by 1,000 bootstrap replications. Sequences cluster in four homologous groups according to the phylogenetic position of their hosts

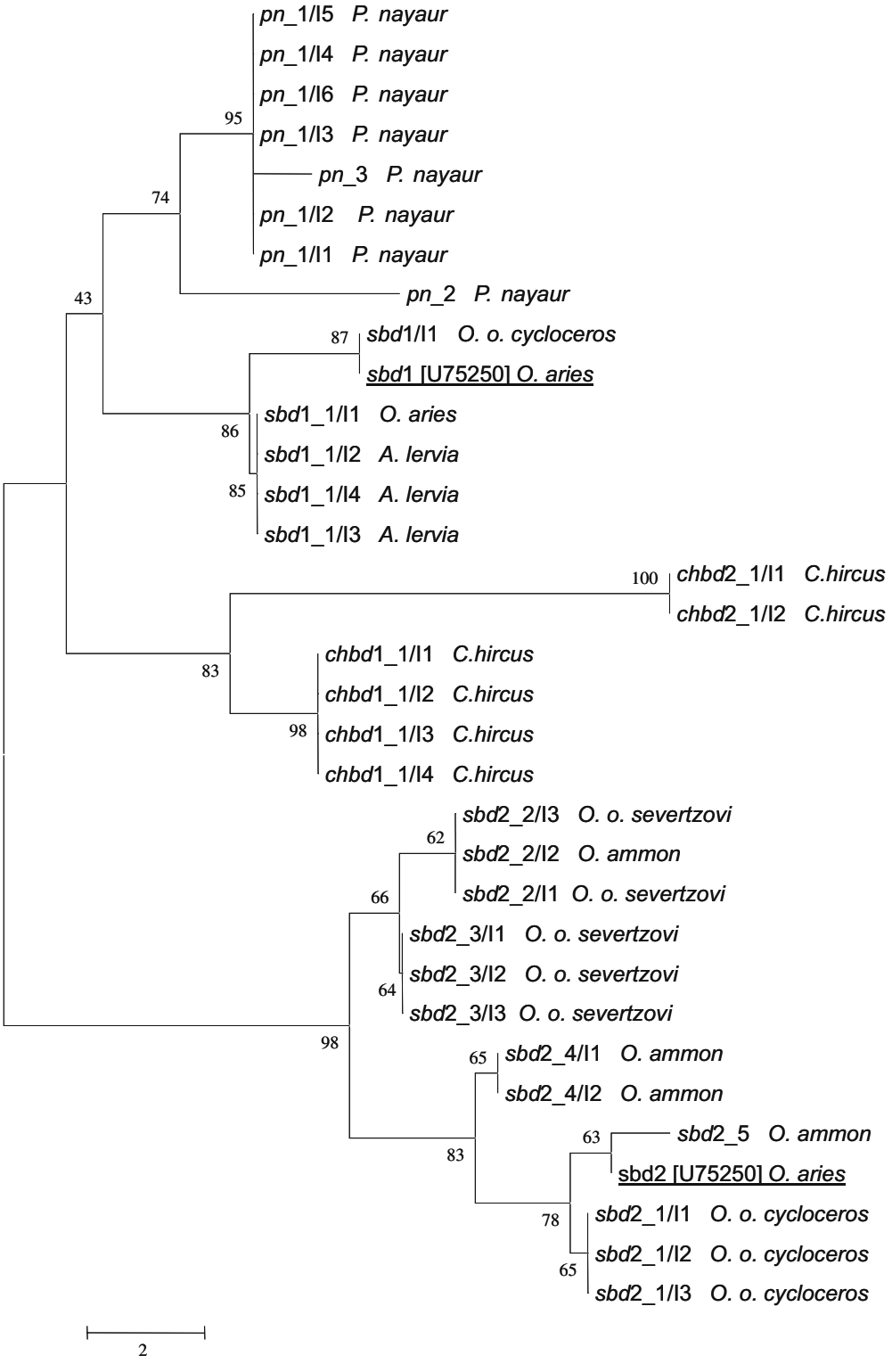

Fig. 3 NJ tree based on proportion difference of nucleotides, using exclusively the exon sequence information. The reliability of each branch was tested by 1,000 bootstrap replications

Using the hierarchical likelihood ratio tests (hLRTs) implemented in MODELTEST (Posada and Crandall 1998), the JC+Γ model of evolution was determined to fit the data set best. It consists of the equal-base frequency model of Jukes and Cantor (1969) combined with among-site rate variation reflected by a gamma (Γ) shape distribution parameter of α=0.1456. The likelihood of the JC+Γ model was −lnL=584.93. None of the tests of neutral evolution (HKA, *D*, *D** and McDonald–Kreitman) found significant deviations from the null hypotheses of non-neutral evolution (Table 3). Thus, we tested whether positive or negative selection acted on the defensin genes by estimating dS and dN substitutions. By grouping homologous sequences as indicated in the NJ tree (Fig. 2), we computed dN and dS

Table 3 Application of four discrete tests of neutral evolution (HKA, *D*, *D**, and McDonald–Kreitman)

Statistical method	*P* value	Deviation from null hypothesis
Fu and Li's *D** test	>0.1	Not significant
Tajima's *D* test	>0.1	Not significant
HKA test		
Capra vs *Ovis*	0.9978	Not significant
Capra vs *Pseudois*	0.6537	Not significant
Ovis vs *Pseudois*	0.8869	Not significant
McDonald–Kreitman test		
Capra vs *Ovis*	1.0000	Not significant
Capra vs *Pseudois*	1.0000	Not significant
Ovis vs *Pseudois*	1.0000	Not significant

None of these tests found significant deviations from the null hypotheses of non-neutral evolution

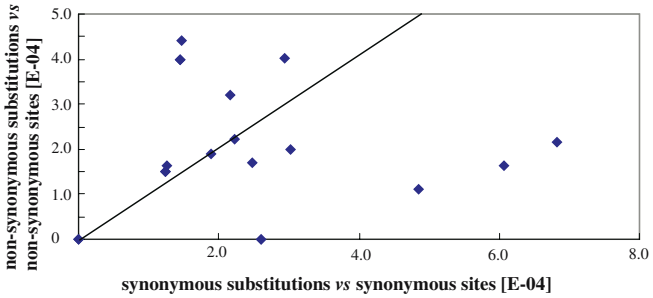

Fig. 4 Plots of numbers of non-synonymous substitutions per non-synonymous site (dN) vs synonymous substitutions per synonymous site (dS) in pairwise comparisons of homologous defensins within Caprini. The *line* marks dN=dS; *symbols* above that *line* indicate dN exceeding dS

for all pairwise comparisons within the four species-specific lineages: (1) *Ovis* defensins, (2) *Capra* defensins, (3) *Pseudois* defensins and (4) *Ammotragus* defensins (Table 4 and Fig. 4). In the case of *Ammotragus* defensins, we found no substitutions, although differences within the intron revealed the existence of at least three sequences. Substitution patterns of *Pseudois* defensins indicated negative selection due to dN values being lower than those of dS. Comparison of substitutions within ovine defensins showed positive selection if the comparison included all ovine sequences (Table 4). Analysis of substitutions referring to single genes indicated that some ovine defensins are under negative selection (Table 4).

Finally, we examined the extent of constraint at the amino acid level of the presented defensins. Alignment of deduced amino acid sequences (Fig. 5) showed conservation of the signal peptide and the six-cysteine array within the mature peptide. Signal peptides were found to be hydrophobic and rich in leucines, with six and seven leucines, respectively, distributed over a length of 19 amino acids. The signalpeptides of all defensins were identical with the exception of *chbd*2, *chbd*2_1 and *chbd*1, differing at one, respectively two sites carrying substituents with similar features (Fig. 5). The mature defensins are positively charged due to the presence of up to five lysines and seven arginines within a range of 38 amino acids. In addition, the six-cysteine motif, as well as the residue positions 47–53, were conserved. All together, we found 22 out of the 38 sites of the mature peptide to be conserved. Three ad-

Table 4 Values of synonymous and non-synonymous substitutions within a genus comparison of defensins from the genera *Ammotragus*, *Capra*, *Ovis* and *Pseudois*

	dS per synonymous site	dN per non-synonymous site
Ovis defensins		
All vs all	2.00E−04±0.000	3.02E−04±0.000
*sbd*1 vs all	2.15E−04±0.000	6.83E−04±0.000
*sbd*2 vs all	1.71E−04±0.000	2.48E−04±0.000
*sbd*1_1 vs all	1.64E−04±0.000	6.07E−04±0.000
*sbd*2_1 vs all	**3.19E−04±0.000**	**2.16E−04±0.000**
*sbd*2_2 vs all	**1.65E−04±0.000**	**1.27E−04±0.000**
*sbd*2_3 vs all	**1.50E−04±0.000**	**1.23E−04±0.000**
*sbd*2_4 vs all	**1.90E−04±0.000**	**1.89E−04±0.000**
*sbd*2_5 vs all	2.23E−04±0.000	2.23E−04±0.000
*sbd*2_3 vs *sbd*2_1	**1.50E−04±0.000**	**1.23E−04±0.000**
*sbd*2_3 vs *sbd*2_4	**3.99E−04±0.000**	**1.45E−04±0.000**
*sbd*2_2 vs *sbd*2_5	**4.02E−04±0.000**	**2.94E−04±0.000**
*sbd*2_2 vs *sbd*2_1	**3.99E−04±0.000**	**2.45E−04±0.000**
Capra defensins		
All vs all	0.00E+00±0.000	2.60E−04±0.000
Pseudois defensins		
All vs all	1.10E−04	4.86E−05
*pn*_2 vs *pn*_1	**4.40E−04**	**1.47E−04**
*pn*_2 vs *pn*_3	**4.40E−04**	**1.97E−04**
Ammotragus defensins		
All vs all	0.00E+00±0.000	0.00E+00±0.000

Values of dS exceeding dN indicating negative selection are in bold

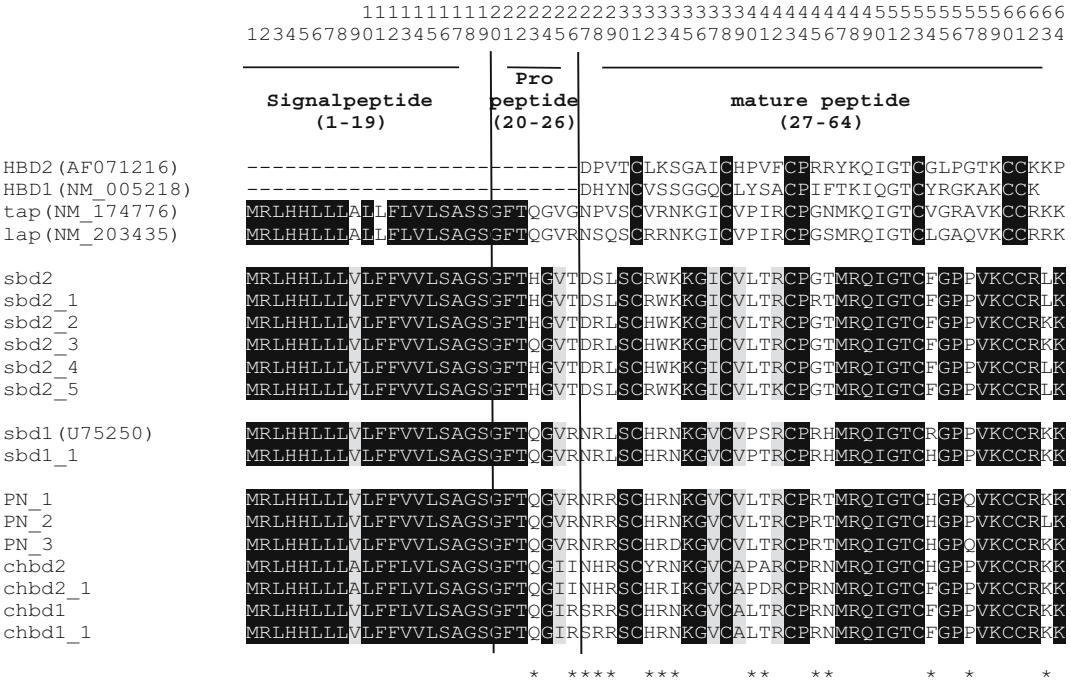

Fig. 5 Multiple sequence alignment of the deduced amino acid sequences of caprine and ovine β-defensins. Sites found to be strictly conserved among Caprini are marked by *black boxes*. Sites with moderate conservation are marked by *grey boxes*. Changes resulting in substituents with different features are indicated by *stars* below the alignment. Conserved sites also identical within, human and bovine defensins are also labelled by *black boxes*. Locations of the signal peptide, the mature peptide and the intervening region of the propiece are depicted

ditional sites of the mature peptide show moderate conservation (substitution of residues with similar physico-chemical features). The remaining 13 sites are characterised by radical non-synonymous changes (Fig. 5).

Discussion

In this study, we investigated the variability and evolutionary relatedness of β-defensins of the tribe Caprini. Due to the lack of genome sequence data bank information which would allow a computational search, we screened the genome via PCR. Our genomic search revealed 13 discrete sequences, including two already known from *O. aries*: *sbd*1 (U75250) and *sbd*2 (U75251). Comparison of newly detected sequences with GenBank entries revealed a high sequence similarity to previously known defensins from *O. aries* [*sbd*1 (U75259) and *sbd*2 (U75251)] and *C. hircus* [*chbd*1 (Y17679) and *chbd*2 (AJ009877)]. For this reason, we classified all sequences detected in species belonging to the genera *Ovis* and *Capra* as variants of either *sbd*1 and 2 or of *chbd*1 and 2. Defensins from *P. nayaur* showed also remarkable sequence similarity, but due to their distinct origin, we classified them as a new group and named them according to their host, *P. nayaur* defensin 1, *P. nayaur* defensin 2 and *P. nayaur* defensin 3 (*pn*_1, *pn*_2 and *pn*_3).

Interestingly, although the tribe Caprini shows a broad spectrum of discrete and diversified defensins, we identified three orthologues showing sequence identity. Orthologues are genes derived from a speciation event, thus they are present in discrete species and have a common ancestor (in contrast to paralogues which are derived by duplication within single individuals). The first one was *sbd*1, which, previously known from *O. aries*, was detected in this study in *O. orientalis severzovi*, the second was *sbd*2_2, detected in *O. ammon* and *O. orientalis severtzovi* and the third one was *sbd*1_1, found in *O. aries* and *A. lervia* (Table 2). The existence of orthologous β-defensin genes, being identical in their coding regions, was already demonstrated for three bovine species (*Bos taurus*, *Bos frontalis* and *Bos javanicus*) (Luenser and Ludwig 2005). Highly similar or even identical genes, distributed over different species, may provide evidence for a common ancestry of these genes.

However, to understand the relationships of the presented defensin genes, we calculated a NJ tree based on their intron sequences. The resulting gene tree groups all sequences into four clearly distinct clusters, with each cluster composed of numerous genes belonging to one taxon (*Ovis*, *Capra*, *Pseudois* and *Ammotragus*). According to the branching order, we propose the existence of a common ancestral β-defensin gene, which became subject to lineage-specific evolution, leading to the ancestral defensins of the genus *Ammotragus* as well as an ancestral defensin common for the genera *Ovis*, *Capra* and *Pseudois*. Subsequent speciation resulted in the divergence of defensins of *Ovis* from *Capra* and *Pseudois*, followed by an additional split into the lineages of *Capra* and *Pseudois*. After diversification of these distinct lineages, the ancestral defensins duplicated repeatedly and independently in each lineage/genus. As a result of this scenario, the detected defensins were distributed species-specifically in numerous copies. As mentioned above, our phylogenetic analyses were based on the intron sequences. Comparison restricted to the coding regions will result in an underestimation of the number of β-defensin genes due to the existence of strongly conserved orthologues and paralogues. In detail, we detected conserved paralogous defen-

sins in *P. nayaur* (*pn*_1/I1–I6), *C. hircus* (*chbd*1_1/I1–I4; *chbd*2_1/I1 and I2), *O. orientalis severtzovi* (*sbd*2_2/I1 and I3; *sbd*2_3/I1–I3), *O. orientalis cycloceros* (*sbd*2_1/I1–I3) and *O. ammon* (*sbd*2_4/I1 and I2). Therefore, we postulate numerous duplication events within certain species; for instance, duplication of an ancestral *C. hircus* defensin resulted in *chbd*1 and *chbd*2. Both genes duplicated independently, leading to four variants of *chbd*1 (chbd1_1/I1, *chbd*1_1/I2, *chbd*1_1/I3 and *chbd*1_1/I4) and two variants of *chbd*2 (*chbd*2_1/I1 and *chbd*2_1/I2), only distinguished by their introns. Thus, we assume that at least four duplication events occurred within *C. hircus*. Genesis of the whole array of β-defensins within the genera *Ammotragus*, *Pseudois* and *Ovis* occurred most likely in a similar way.

Interestingly, defensin genes of *Pseudois* are more closely related to those of *Capra* than to those of *Ovis*. The phylogenetic position of *Pseudois* has long been discussed controversially; because of the existence of both goat- and sheep-like features, *Pseudois* was often referred to as an intermediate. Comparison of the 12S rDNA sequences grouped *Pseudois* together with *Capra*, separated from *Ovis* (Ludwig and Fischer 1998). Resembling these findings, our gene tree is in agreement with the current opinion of the Caprini phylogeny.

In conclusion, the gene tree clearly indicates a homology of all caprine defensins and provides evidence for a lineage-specific evolution. Homologous genes were duplicated repeatedly and independently within each lineage. Redundant gene copies enabled accumulation of mutations by chance; such mutations may endow this gene with a new function (Ohno 1973). Diversification may reflect species-specific adaptation to the local environment or ecological niches. In addition, certain duplicates seem to be strongly conserved (*sbd*2_1, *sbd*2_2, *sbd*2_3 and *sbd*2_4; *chbd*2_1, *chbd*1_1, *sbd*1_1 and *pn*1_1).

Gene duplication is regarded to be a key step for the creation of new genes and genes with a new function (Ohno 1970). Due to the prevalence of duplicate genes in genomes of all three domains of life (archaebacteria, bacteria and eucaryotes), the idea of gene duplication has risen to a general principle in evolutionary biology. Examples for duplicated defensins were provided by numerous studies (Hughes 1999; Lynn et al. 2004; Maxwell et al. 2003).

However, while in Ohno's (1970) view, the duplicate may gain a new function (neofunctionalisation), balanced by the other which is retaining the ancestral function, our study provides evidence not only for neofunctionalisation but also for maintaining identical duplicates. Redundancy may directly be advantageous as a mechanism for compensation of deleterious mutations (Clark 1994) and/or developmental accidents (Nowak et al. 1997). In addition, redundancy enables the generation of large amounts of gene products (Zhang 2003). This idea of redundancy being beneficial by providing large amounts of transcripts is supported by the fact that β-defensins are synthesised de novo upon stimulation and not stored like α-defensins (Brockus et al. 1998; Russell et al. 1996; Schonwetter et al. 1995). Yet there is a problem: if a gene is truly redundant, it would not be protected against an accumulation of deleterious mutations, which would lead to a purifying selection of that copy. Therefore, unless the presence of an extra amount of gene product may be advantageous, two genes with identical functions are unlikely to be stably maintained in the genome (Nowak et al. 1997). Theoretical population genetics predicts that both duplicates can be maintained when they differ in some aspects of their function (Nowak et al. 1997). Other than Ohno's (1970) model of neofunctionalisation, differing function can also be reached by division of gene expression after gene duplication (subfunctionalisation) (Force et al. 1999). Detection of two isoforms of a goat defensin expressed specifically in digestive and respiratory tissues (Zhao et al. 1999) may represent such a process of subfunctionalisation.

Comparison of both trees (intron tree and exon tree) depicts the poorly resolved deep branches of the exon tree. The distinct phylogenetic origin of certain defensins, as retrieved from the intron tree, is only poorly reflected and lost. For example, all variants of *sbd*1_1 representing defensins of the two distinct genera *Ovis* and *Ammotragus* are grouped together and close to the ovine defensins *sbd*1 from domestic sheep and *sbd*1/I1 from argali sheep, although early speciation separated ancestral *Ammotragus* defensins (*sbd*1_1/I2, *sbd*1_1/I3 and *sbd*1_1/I4) from all the others (Fig. 2). In addition, certain species-specific duplication events are not reflected in the tree due to exon identity (*pn*_1, *chbd*1_1, *chbd*2_1, *sbd*2_1, *sbd*2_2, *sbd*2_3 and *sbd*2_4). Obviously, certain defensins are strongly conserved in their coding regions, both intra- as well as interspecifically; therefore, detection of lineage-specific separation of an ancestral defensin and their independent duplication events within distinct lineages requires the information stored in the introns.

However, resuming the branching order of the gene trees, we found evidence for diversifying positive selection, as indicated by the broad array of discrete defensins within a common species, as well as conservation indicated by the existence of identical exons distributed inter- and intraspecifically. To understand the underlying mode of evolution, we first tested for the neutral mode of evolution. Several synonymous nucleotide changes were detected within caprine and ovine defensin genes. The fixation of synonymous substitutions conforms to the neutral theory of molecular evolution (Kimura 1977) and is expected for most genes (Nei 1987). However, the application of a statistical test checking for the neutral mode of evolution clearly demonstrated that the observed variations did not fit that model (Table 3). Therefore, the presence of selection pressure was assumed. Subsequently, we tested for positive and negative selection via a comparison of synonymous and non-synonymous substitutions. In some gene comparisons, the rate of synonymous substitutions exceeded the rate of non-synonymous substitutions. This was the case for all defensins of *C. hircus* and *P. nayaur*, indicating that these genes did not evolve under positive-selection pressure. *A. lervia* defensins showed neither synonymous nor non-synonymous substitutions, a situation which may be regarded as a special case of negative selection. Compar-

ison of ovine defensins revealed positive selection if all sequences were included. Analysis of discrete genes also indicated that some genes were obviously negatively selected (Table 4). In conclusion, comparison of caprine β-defensins showed both positively as well as negatively selected genes (Table 4 and Fig. 4). Such examples were recently also described by others for defensins evolving under positive (e.g. Hughes 1999; Lynn et al. 2004; Luenser and Ludwig 2005; Maxwell et al. 2003) and negative selections (e.g. Del Pero et al. 2002; Luenser and Ludwig 2005).

Comparison of the deduced amino acid sequences of the mature peptides showed moderate changes, which did not affect the net charge of the peptides. The mature peptide consists of 38 sites, out of which 22 sites were found to be conserved within the Caprini (Fig. 5). These sites included the six cysteines, the region from position 47–52 and some single positions. Thirteen positions within the mature peptide were found to be substituted by amino acids with different features (Fig. 5). It is likely that these changes affected the biological activity, but in which way is unclear. It is known from other defensins that small changes in primary structure can have an enormous effect on their potency. Two human neutrophil peptides (HNP1 and HNP3), which differ by only one amino acid, show drastic differences regarding their potency against *Candida albicans*: While the addition of HNP1 to *C. albicans* results in the yeast's death, that power is totally lost in HNP3 (Lehrer et al. 1988; Raj et al. 2000). A similar observation was made for two mouse α-defensins (cryptdin 4 and cryptdin 6). Variants of cryptdin 4 and cryptdin 6, truncated at the N-terminus for one and two sites, respectively, showed markedly less anti-microbial activity (Ouellette et al. 2000). However, we can only speculate which changes of toxicity or specificity may result from the 13 positions found to be variable within the mature caprine β-defensins. To gain more information, studies about the expression of these newly detected genes need to be carried out. Perhaps, there are differences regarding the tissues where they are expressed, resembling the findings of Zhao et al. (1999), who detected two isoforms of goat defensins expressed specifically in distinct tissues.

In conclusion, we detected a broad array of defensin genes which was shown to be monophyletic and which we suggest to be the result of several rounds of gene duplication events. Comparison of the coding sequences suggested distinct fates of the duplicates. We found evidence of neofunctionalisation, indicated by sequence diversification, as well as evidence for subfunctionalisation, which may explain the conservation of exons. Numerous duplicates may have been lost due to deleterious mutations and purifying selection. However, our data accentuate how important and informative the inclusion of the defensin introns is for the reconstruction of phylogenetic history. Obviously, evolutionarily stably maintained duplicates on one side and their diversification on the other provide evidence for rapid evolution and confirm the importance of the innate immunity despite the existence of the adaptive immunity.

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