Published in final edited form as:
Comp Biochem Physiol Part D Genomics Proteomics. 2009 March 1; 4(1): 11–20. doi:10.1016/j.cbd.2008.09.002.

Bovine Carboxylesterases: Evidence for Two CES1 and Five Families of CES Genes on Chromosome 18

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Abstract

Predicted bovine carboxylesterase (CES) protein and gene sequences were derived from bovine (*Bos taurus*) genomic sequence data. Two bovine *CES1* genes (*CES1.1* and *CES1.2*) were located on chromosome 18 encoding amino acid sequences that were 81% identical. Two forms of CES1.2 were also observed apparently caused by an indel polymorphism encoded at the C-terminus end. Two *CES* gene clusters were observed on chromosome 18: *CES5-CES1.1-CES1.2* and *CES2-CES3-CES6*. Bovine CES1, CES2, CES3, CES5 and CES6 shared 39-45% identity with each other, but showed 71-76% identity with each of the five corresponding human CES family members. Phylogeny studies indicated that bovine *CES* genes originated from five ancestral gene duplication events which predated the eutherian mammalian common ancestor. In addition, a subsequent *CES1* gene duplication event is proposed during mammalian evolution prior to the appearance of the Bovidae common ancestor ~ 20 MY ago.

Keywords

Bos Taurus; Mammals; Genome; carboxylesterase; CES; evolution; gene duplication

Introduction

Many drugs, pro-drugs, xenobiotics, narcotics and pro-herbicidal esters are metabolized by carboxylesterases (CES; E.C.3.1.1.1) (Satoh & Hosokawa, 1998; Satoh et al., 2002; Ohtsuka et al.,2003; Redinbo and Potter, 2005; Gershater et al.,2006). CES also detoxifies insecticides, carbamates and organophosphates (Ahmad & Forgash, 1976; Leinweber, 1987), catalyses several lipid metabolism reactions (Ghosh, 2000; Tsujita and Okuda, 1993; Becker et al., 1994; Hosokawa et al.,2007; Diczfalusy et al., 2001) and the conversion of lung alveolar surfactant (Ruppert et al., 2006), and may participate in the assembly of liver lipoprotein particles (Wang et al.,2007).

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Five families of mammalian CES have been reported (Holmes et al., 2008a,b). These include CES1 (Munger et al., 1991; Shibata et al. 1993; Ghosh 2000; Holmes et al., 2008c; Gene Card CES1, 2008) and CES2 (Langmann et al. 1997; Schewer et al. 1997; Holmes et al., 2008c; Gene Card CES2, 2008), the major enzymes in mammalian liver and intestine, respectively; CES3 (Sanghani et al. 2004; Gene Card CES3, 2008), expressed in human liver, colon and brain; CES5 (or CES7), a major urinary protein of the domestic cat (Miyazaki et al. 2003; 2006; Holmes et al., 2008b; Gene Card CES7, 2008); and CES6 (or ESTHL), a predicted subunit expressed in human brain (Clark et al. 2003). Human *CES4* is an apparent pseudogene member of the mammalian CES1 family (Yan et al., 1999; Gene Card CES4, 2008). The structure-function relationships for human CES1 have been examined by three dimensional studies and three ligand binding sites reported: the active site, the 'Z-site' and the 'side door', where substrates, cholesterol analogues and acyl groups are bound, respectively; and a 'gate', which may regulate product release (Bencharit et al., 2003; 2006; Fleming et al., 2005).

The structures for human (Becker et al.,1994; Langmann et al., 1997; Ghosh, 2000; Marsh et al., 2004) and rodent *CES1* and *CES2* 'like' genes (Ghosh et al.,1995; Dolinsky et al., 2001; Hosokawa et al.,2007) have been determined. In addition, predicted *CES* gene structures have been described for five families of mammalian *CES* following the release of several mammalian genome sequences (Holmes et al., 2008a,b). The genome sequence of domestic cattle has been reported (Bovine Genome Project, 2008) providing an opportunity for *in silico* interrogation and analyses of bovine genes and proteins. This paper describes predicted gene and amino acid sequences and secondary structures for bovine CES1, CES2, CES3, CES5 and CES6, as well as biochemical, phylogenetic and evolutionary relationships for these enzymes. Bovine liver CES has been purified and kinetically characterized (Runnegar et al., 1969; Stoops et al., 1975), and several bovine CES GenBank mRNA sequences have been reported (see Table 1), however there have been no reports concerning bovine CES gene structures and functions.

The domestic beef and dairy cattle industries make major contributions to the economies of many countries, with industry revenues amounting to US$36.7 and US$26.7 billion respectively, for the United States alone (IbisWorld Industry Reports, 2008). Domestic cattle also contribute to nutrition through human and animal consumption of meat and dairy products. Many veterinary drugs used in pain management in cattle or during surgery contain ester or amide moieties, such as procaine, aspirin, peroxicam, paracetemol and phenacetin (Anderson and Muir, 2005; Gentili, 2007), and are likely to be metabolized by CES. Consequently, this study of the genomic and protein structures and proposed functions for bovine CES will be of considerable interest and significance.

Methods

In silico Bovine CES Gene and Protein Identification

Amino acid and cDNA sequences for various forms of bovine (CES1, CES2 and CES6) and human (CES1, CES2, CES3, CES5 and CES6) CES were obtained from UniProtKB/Swiss-Prot [http://au.expasy.org] and GenBank [http://www.ncbi.nlm.nih.gov/Genbank/] database sources (see Table 1). For bovine CES3 and CES5, BLAST interrogations were undertaken using human CES3 and CES7 protein sequences [http://au.expasy.org] and NCBI web tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to interrogate the non-redundant protein sequences database for *Bos taurus.* A predicted bovine CES3 sequence was generated using the blast-p program algorithm, whereas a bovine CES5 sequence was derived from an annotated genomic sequence (NW_932181) using the gene prediction method: GNOMON to interrogate the database 'build protein' with a BLASTP program (see Table 1).

Gene locations, predicted gene structures and CES protein subunit sequences were observed for each CES examined for those regions showing identity with the respective bovine *CES* gene products using the UC Santa Cruz web browser [http://genome.ucsc.edu/cgi-bin/hgBlat] (Kent et al., 2002) with the default settings. In addition, predicted gene sequences were obtained for exons 13 and 14 and intron 13 for two CES1-like genes (designated *CES1.1* and *CES1.2*) and two CES *CES1.2* variant genes (designated as *CES1.2A* and *CES1.2B*) using the respective amino acid sequences to interrogate the bovine genome and investigate the genetic distinctness for the two predicted *CES1* genes.

Predicted Secondary Structures for Bovine CES Subunits

Predicted secondary structures for bovine CES1 (subunits 1.1, 1.2A and 1.2B), CES2, CES3, CES5 and CES6 were obtained using the PSIPRED v2.5 web site tools provided by Brunel University [http://bioinf.cs.ucl.ac.uk/psipred/psiform.html] (McGuffin et al.,2000).

Phylogenetic Studies and Sequence Divergence

Phylogenetic trees were constructed using an amino acid alignment from a ClustalW2-derived alignment of CES protein sequences, obtained with default settings and corrected for multiple substitutions (Larkin et al., 2007) [http://www.ebi.ac.uk/clustalw/]. Alignment ambiguous regions, including the amino and carboxyl termini, were excluded prior to phylogenetic analysis yielding alignments of 483 residues of human and bovine CES1, CES2, CES3, CES5 and CES6 sequences (Table 1). Pairwise scores were calculated using the number of identities in the best alignment divided by the number of residues compared. Scores were initially calculated as percent identity scores and were converted to distances by dividing by 100 and subtracting from 1.0 to give the number of differences per site. The extent of divergence for the human and bovine CES1, CES2, CES3, CES5 and CES6 subunits were determined using the SIM-Alignment tool for Protein Sequences [http://au.expasy.org/tools/sim-prot.html] (Pietsch, 1995; Schwede et al.,2003).

Results

Alignments of Predicted Bovine CES1 Amino Acid Sequences with Human CES1

The deduced amino acid sequences for two distinct bovine CES1 subunits (designated as CES1.1 and CES1.2) are shown in Figure 1 with variant sequences for bovine CES1.2 (designated as CES1.2A and CES1.2B) and human CES1 (Munger et al., 1991;Shibata et al., 1993;Gene Card CES1, 2008) (Table 1). Alignments of these CES subunits showed 71 and 76% sequence identities for bovine CES1.1 and CES1.2B, respectively (Table 2), indicating that these protein subunits are products of the same *CES* gene family. The amino acid sequences for bovine CES1 subunits were one (bovine CES1.2B) or three (bovine CES1.1) residues shorter than for human CES1 (567 residues) largely due to differences in the lengths of the N-terminus signal peptide (Figure 1). Comparisons of bovine CES1 sequences with human CES1 enabled identification of key residues which may contribute to catalysis, ligand binding, quaternary structure and regulatory functions: the catalytic triad for the active site (Ser228; Glu345; His458) (human CES1 residue numbers are used) (Cygler et al.,1993); microsomal targeting sequences, including the hydrophobic N-terminus signal peptide (von Heijne, 1983;Zhen et al.,1995;Potter et al., 1998) and the C-terminal endoplasmic reticulum (ER) retention sequence (His-Ile-Glu-Leu) (Robbi and Beaufay, 1983; Munro and Pelham, 1987;Zhen et al.,1995); disulfide bond forming residues (Cys95/Cys123 and Cys280/Cys291) (Lockridge et al.,1987); and ligand binding sites, including the ‘Z-site’ (Gly358), the ‘side door’ (Val424-Met425-Phe426) and the ‘gate’ (Phe551) residues (Bencharit et al., 2003, 2006). Identical sequences, or conservative substitutions, were observed for the two bovine CES1 subunits for the above key residues, with the exception of the ‘side-door’ for bovine

CES1.1, which retained only two of the bulky amino acid residues (Leu422-Phe423) observed for human CES1 (Val424-Met425-Phe426).

Other key human CES1 sequences included two charge clamps which are apparently responsible for subunit-subunit binding, namely residues Lys78/Glu183 and Glu72/Arg193 (Bencharit et al.,2003, 2006; Fleming et al., 2005), which have been retained for both bovine CES1 subunits (Figure 1). The N-glycosylation site for human CES1 at Asn79-Ala80-Thr81 (Kroetz et al., 1993; Bencharit et al., 2003; 2006; Fleming et al., 2005) has been retained for bovine CES1.1 (Asn78-Thr79-Thr80) but not for bovine CES1.2, with Thr80 being replaced by Ile80 (Figure 1; Table 2). Another potential N-glycosylation site was observed however for bovine CES1.1 (491Asn-492Leu-Ser493) and CES1.2.B (490Asn-491Leu-492Ser) (Table 2).

Two nearly identical CES1.2 sequences were observed which differed significantly at the C-terminus, with CES1.2A being 7 residues shorter and lacking the endoplasmic reticulum targeting sequence reported for human CES1 (His564-Ile565-Glu566-Leu567) (Robbi and Beaufay, 1991). The nucleotide and deduced amino acid sequences for this region of bovine CES1.2A and CES1.2B showed that a 4 nucleotide deletion has contributed to this change in C-terminal sequence for CES1.2A by introducing a stop codon at an earlier termination site (Figure 2).

Alignments of Bovine CES2, CES3, CES5 and CES6 with Human CES Amino Acid Sequences

The deduced amino acid sequences for bovine CES2, CES3, CES5 and CES6 are shown in Figure 3 together with the previously reported sequences for human CES2 (Schewer et al., 1997;Pindel et al., 1997;Gene Card CES2, 2008); human CES3 (Clark et al., 2003;Sanghani et al., 2004;Gene Card CES3, 2008); human CES5 (Ota et al., 2004;Gene Card CES7, 2008); human CES6 (Ota et al., 2004); and human CES1 (Munger et al., 1991;Shibata et al., 1993;Gene Card CES1, 2008) (see Table 1). The alignments of predicted bovine CES amino acid sequences with human CES1 identified several key residues, including the active site ‘triad’, the hydrophobic N-terminus signal peptide and the disulfide bond forming residues, however only bovine and human CES2 and CES3 sequences contained the C-terminal endoplasmic reticulum retention sequences, namely HTEL (human and bovine CES2), QEDL (human CES3) and QEEL (bovine CES3). Bovine and human CES5 and CES6 C-terminal sequences lacked the endoplasmic reticulum retention tetrapeptide sequence, with CES5 showing high content of hydrophobic amino acids for an additional 12 amino acids concluding with an Ala-Pro sequence in each case (Figure 3). Bovine CES2, CES3 and CES6 sequences lacked the human CES1 N-glycosylation site at Asn79-Ala80-Thr81, but exhibited other potential N-glycosylation binding sites: bovine CES2 with 2 such sites: Asn109-Val110-Thr111 and Asn274-Leu275-Ser276; bovine CES3, one site at Asn212-Asn213-Ser214; and bovine CES6, one site at Asn375-Val376-Thr377. Bovine CES5 however not only retained the human CES1 N-glycosylation site (Asn84-Ala85-Thr86) but exhibited 3 other potential sites at Asn361-Lys362-Ser363, Asn517-Ile518-Ser519 and Asn517-Ile 518-Ser519 (Table 3). Charge clamp residues reported for human CES1 were absent in the predicted bovine CES2, CES3 and CES5 sequences however one of the potential charge clamps was retained for bovine CES6 (73Glu..186Arg) (Figure 3). The ‘Z-site’ residue (Gly358 for human CES1) was retained for bovine CES2, CES3 and CES5 sequences but replaced in bovine CES6 (Asn355), whereas ‘side-door’ residues (Val424-Met425-Phe426 for human CES1) have undergone conservative substitutions for bovine CES3 (Ile415-Ile416-Ile417) and bovine CES5 (Val410-Phe411-Phe412), but reduced in length to two hydrophobic residues for bovine CES2 (Leu409-Phe410) and CES6 (Ala422 and Phe424). The ‘gate’ residue for human CES1 (Phe551) has undergone a conservative substitution for all of the predicted bovine CES sequences (Leu for bovine CES2, CES3, CES5 and CES6 sequences).

Sequence identities for bovine and human CES1, CES2, CES3, CES5 and CES6 showed that the subunits showed higher levels of identities within families (71-76%) whereas CES subunits from different families exhibited lower levels of sequence identity (39-46%) (Table 2). This supports the proposal that human and bovine CES2, CES3, CES5 and CES6 subunits represent enzymes derived from different families in each case. Human and bovine CES6 sequences however shared 15 additional residues with human and bovine CES1 (residues 308-322 of human CES1) in comparison with the human and bovine CES2, CES3 and CES5 sequences (Figures 1 and 3).

Predicted Secondary structures for Bovine CES Isozymes

Analyses of predicted secondary structures for two bovine CES1 subunits, and for bovine CES2, CES3, CES5 and CES6 subunits, were compared with the previously reported secondary structure for human CES1 (Bencharit et al., 2003, 2006) (Figures 1 and 3). Similar α-helix β-sheet structures were observed for all of the bovine and human CES subunits examined and comparable structures were predicted near key residues including the α-helix within the N-terminal signal peptide; the β-sheet and α-helix near the active site Ser228 (human CES1) and 'Z-site' (Glu354/Gly356 respectively); the α-helices bordering the 'side door' site; and the α-helix containing the 'gate' residue (Phe551 for human CES1). In addition, two random coil regions (residues 51-115 and 169-188 for human CES1) were predominantly retained for all bovine CES forms examined. Comparable human CES1 regions contained two charge clamps sites (Lys79..Glu183 and Glu73..Arg186); an N-glycosylation site at Asn79-Ala80-Thr81; a potential N-glycosylation site for human and bovine CES2 (Asn109-Val110-Thr111 for bovine CES2), and one of the disulfide bridges (87Cys /117Cys) reported for human CES1. In addition, bovine and human CES5 secondary structures gave an additional helix at the hydrophobic C-termini, in each case.

Predicted Gene Locations and Exonic Structures for Bovine CES1, CES2, CES3, CES5 and CES6 Genes

Table 1 and Figure 4 summarize the predicted locations for bovine CES1, CES2, CES3, CES5 and CES6 genes based upon BLAT interrogation of the bovine genome (Bovine Genome Project, 2008), using the reported sequences for the corresponding human CES sequences and the UC Santa Cruz Web Browser (Kent et al., 2003). All of the predicted bovine CES genes were located on chromosome 18 in two clusters, with the two *CES1* 'like' genes located near to the predicted bovine *CES5* gene. The predicted bovine *CES2*, *CES3* and *CES6* genes were found in a second *CES* cluster located 8.5 million base pairs downstream on chromosome 18. The bovine *CES1.1* and *CES1.2* genes were transcribed on the negative strand whereas bovine *CES2*, *CES3*, *CES5* and *CES6* genes were transcribed on the positive strand. Figure 1 summarizes the predicted exonic start sites for the bovine *CES1.1* and *CES1.2* genes which have 14 exons, in identical or similar positions to those described for the human CES1 gene (Becker et al. 1994;Langmann et al. 1997). Bovine *CES2*, *CES3*, *CES5* and *CES6* genes contained 12, 13, 13 and 14 predicted exons respectively (Figure 3), in similar positions to those observed for the human *CES2* (Tang et al., 2008; Gene Card CES2, 2008), *CES3* (Clark et al., 2003; Gene Bank CES3, 2008), *CES5* (Ota et al., 2004;Gene Card CES7, 2008) and *CES6* genes (Ota et al., 2004), respectively.

Phylogeny and Divergence of Human and Bovine CES Sequences

A phylogenetic tree (Figure 5) was calculated by the progressive alignment of human and bovine CES1, CES2, CES3, CES5 and CES6 amino acid sequences which showed clustering into five main groups in accordance with the proposed *CES* gene family. Two bovine CES1 subunits were grouped together on a separate branch of the CES1 cluster supporting a proposal that these represent CES1 like forms. Table 2 summarizes the percentages of sequence

identities for bovine CES subunits and human CES subunits. Bovine CES1.1 and CES1.2B sequences shared a high level of identity with each other (81%), and with human CES1 (71% and 76%, respectively). The average amino acid sequence divergence rate for mammalian CES1 was calculated using the average genetic distance observed for bovine CES1.1, CES1.2 and human CES1 from the eutherian mammalian CES1 common ancestor and the date of appearance for a common ancestor for these species (84-99 MY ago) (Murphy et al. 2001;Woodburne et al. 2003). An amino acid substitution rate of 0.11-0.13% per million years of mammalian evolution was observed (Table 4), which was then used to estimate the time for the appearance of the common ancestral gene for the bovine *CES1.1* and *CES1.2* genes at 62-73 MY ago (Table 4).

Discussion

The sequencing of the genome of domestic cattle, *Bos taurus*, was a major achievement in modern genetic research which will assist in identifying key features of the mammalian genome and provide tools and data for researchers to better understand bovine molecular evolution, genetics, biochemistry and disease (Bovine Genome Project, 2008). Knowledge of the bovine genome sequence will also assist in the mapping of specific diseases within modern breeds; contribute to a better understanding of important areas of human health such as obesity, female health and communicable diseases; and provide a resource to study genetic and phenotypic diversity within the hundreds of cattle breeds developed for meat and dairy production and for assistance with labor (Purdy et al., 2008).

Although mammalian CES may serve a variety of functions, the enzyme has been most extensively investigated with respect to its role in drug metabolism, particularly the first-pass clearance or modification of drugs by the intestine and jejunum following ingestion (Imai, 2006); the first pass clearance of inhaled drugs in the lung (Imai et al., 2003); and the clearance of drugs from the body by liver CES following absorption into the circulation (Satoh & Hosokawa, 1998; Satoh et al.,2002). Many common drugs are used to improve health, treat disease and control pain in both humans and cattle (Anderson and Muir, 2005; Gentili, 2007). Given the diversity of roles for CES, including lung surfactant formation (Ruppert et al., 2006), xenobiotic, insecticide, lipid and cholesterol metabolism (Ahmad & Forgash, 1976; Tsujita et al.,1993; Becker et al., 1996) and pheromone metabolis m (Miyazaki et al., 2005), studies of the genetics and biochemistry of bovine CES will contribute to an improved understanding of these metabolic processes in cattle, and of associated diseases.

Bovine liver CES has been subjected to large scale purification and to biochemical and kinetic analysis using a range of substrates, with a subunit weight of 70,000 based on titration with p-nitrophenyl dimethylcarbamate (Runnegar et al. 1969; Stoops et al.1975). There are no reports however of amino acid sequences for bovine CES with the exception of those deduced from mRNA sequences for bovine CES1.1, CES1.2A, CES1.2B, CES2 and CES6 (see Table 1). In addition, there are no genetic analyses of bovine CES reported in the literature (Fries & Ruvinsky, 2005).

Major structural features for human CES1 have been described in detail by X-ray crystallographic methods (Bencharit et al.,2003; 2006; Fleming et al., 2005). The enzyme has three functional domains, including the catalytic domain with the carbohydrate binding and triad of active site residues; the αβ domain comprising the hydrophobic internal structure and forming the subunit-subunit binding sites for this enzyme; and the regulatory domain facilitating substrate binding, product release and the trimer-hexamer equilibrium. Several amino acid residues have been strictly conserved for bovine CES1.1 and CES1.2 in key sites identified for human CES1, including the active site ‘triad’, the cholesterol-like binding Z-site (Gly467), the disulfide bonds, the hydrophobic N-terminus signal peptide (residues 1-16) and

the C-terminal endoplasmic reticulum retention sequence His-Val-Glu-Leu (residues 561-564). The latter sequence apparently functions in CES retrieval from the Golgi apparatus and retention by the ER lumen (Munro and Pelham, 1987; Robbi and Beaufay, 1991; Potter et al., 1998). The bovine CES1.2A C-terminus lacks this sequence however which may alter the distribution characteristics for this enzyme. Miller and coworkers (1999) have described a bioengineered form of human CES1 for which the His-Ile-Glu-Leu was changed with a replacement Arginine at the C-terminus. This resulted in the secretion of this enzyme from human 293T cells in comparison with the native enzyme which was retained within the endoplasmic reticulum. It is likely then that bovine CES1.2A may serve as a secreted form *in vivo*, which may influence its biochemical role in the body.

Other conserved bovine CES1 residues which may also reflect functions reported for human CES1 (Bencharit et al.,2003; 2006; Fleming et al., 2005). These include the N-glycosylation binding site (Asn79-Ala80-Thr81) (Kroetz et al.,1993) which was found in bovine CES1.1 (Asn78-Thr79-Thr80) but not in bovine CES1.2. All three forms of bovine CES1 however exhibited another potential N-glycosylation binding site (Asn488-Leu489-Ser490) which may participate in further carbohydrate binding at this site (Table 3). The charge clamps for human CES1 (charge clamps 1 [Glu72/Arg186] and 2 [Lys78/Glu183] which support the oligomeric subunit structure for this enzyme have been retained by the bovine CES1 subunits, suggesting that bovine CES1 subunits also form oligomers. The human CES1 cholesterol analogue binding 'Z' site (Gly356) was also present in bovine CES1.1 and CES1.2 although the 'side door' to the human CES1 active site (Val424-Met425-Phe426) was reduced to two hydrophobic residues for bovine CES1.1 (Leu422-Phe423) as compared with bovine CES1.2. The active site 'gate' for human CES1 (Phe551) was conservatively substituted for bovine CES1.1 and CES1.2 (Leu). We have concluded that bovine CES1 subunits have retained the major features reported for human CES1, but with some changes. Bovine CES1.2A is likely to be secreted from bovine tissues, and the smaller hydrophobic 'side door' for bovine CES1.1 may impact on the catalytic properties for this enzyme (Figure 1). There are also changes in the number and position of N-glycosylation sites for bovine CES1 subunits which may influence kinetic and/or stability properties for these enzymes (Table 3).

Bovine liver CES has a multimeric subunit structure (Stoops et al.,1975), which is supported by the retention of subunit-subunit binding sites reported for human CES1 (Figure 1) (Bencharit et al.,2003; 2006; Fleming et al., 2005). In contrast to CES1, human and baboon CES2 are monomeric enzymes (Pindel *et al.* 1997; Holmes et al., 2008c), which is supported by the substitution of residues maintaining the charge clamps for human CES1 (Fleming et al., 2005). The bovine CES2 sequence was consistent with human and baboon CES2, showing that human CES1 charge clamps would not function for the bovine CES2 sequence in the corresponding positions. This predicted monomeric subunit structure for bovine CES2 may also be shared by bovine CES3 and CES5, which also lack the CES1 charge clamp amino acid residues. Bovine CES6, however, retains one of the potential charge clamps sites which may contribute to an oligomeric structure for this enzyme.

Human CES1 functional residues that have been conserved for bovine CES2, CES3, CES5 and CES6 sequences include the active site triad, the disulfide residues and the predicted active site 'gate' (Leu531 for bovine CES2), proposed to assist in the release of acyl groups following hydrolysis (Bencharit et al., 2003; 2006) (Figure 3). Others include the predicted CES 'Z-site' (Gly356 for human CES1), which was retained in bovine CES2, CES3 and CES5 sequences, but not for CES6 (Asn355). Human CES1 'side door' residues have undergone conservative substitutions for bovine CES2, CES3 and CES5, while bovine CES6 has retained only two of these hydrophobic residues. These changes to the active site side door sequences may introduce significant changes in catalysis for the CES classes, in comparison with CES1. It would appear then that bovine CES2, CES3, CES5 and CES6 subunits have retained essential

residues for enzyme catalysis but they are likely to exhibit distinct kinetic properties as a result of the amino acid substitutions in the side door and gate regions.

Bovine CES5 shared a similar 12 amino acid sequence at the C-terminus with human CES5, and both enzymes lacked the endoplasmic reticulum retention sequence reported for human CES1. This is comparable to the C-terminal sequences for other forms of mammalian CES5 examined, with the exception of the cat CES5 C-terminal sequence, which has a reduced length of 544 residues, apparently as a result of a 'stop' codon within the cat CES5 coding sequence (Holmes et al., 2008b). Human CES5 has been described as a secreted enzyme (Clark et al., 2003). This is a property shared with the domestic cat CES5 (also called cauxin for carboxylesterase-like urinary excreted protein or CES7), which is secreted from the epithelial cells of kidney distal tubules, and proposed to function in regulating the production of a pheromone excreted in cat urine (Miyazaki et al.,2003; 2006).

The phylogenetic tree reported here for human and bovine CES subunits (Figure 5) was obtained by the progressive alignment of two predicted bovine CES1 like amino acid sequences (CES1.1 and CES1.2) with human CES1 and the sequences for human and bovine CES2, CES3, CES5 and CES6 (see Table 1). The tree showed six clusters of sequences which were consistent with these CES genes being distinct family groups. This supports previous studies for eutherian and marsupial CES sequences, which proposed a series of rapid gene duplication events between 328 and 378 MY ago which generated the *CES* gene families present today (Holmes et al., 2008a). Using an amino acid substitution rate for mammalian CES1 subunits (Table 4), it is estimated that that the gene duplication event generating the two bovine CES1 'like' genes occurred >60 MY ago. This is prior to the common ancestor for the *Bovidae* family, for which fossils have been reported from the early Miocene period, about 20 MY ago (Vrba, 1985; Mathee & Robinson, 1999). The tandem location for the bovine CES1 genes (Table 1;Figure 3) lends support to an unequal crossover event mechanism for generating the *CES1.1* and *CES1.2* genes on chromosome 18, which is similar to that proposed for hemoglobin and alpha-satellite genes (Metzenberg et al., 1991;Alkan et al., 2004). Comparable *CES1* gene duplication events have also occurred in the mouse, where four CES1 like genes are closely linked on chromosome 8 (Berning et al., 1985;Furihata et al., 2003;2005;2006;Hosokawa et al.,2007).

In conclusion, the results of the present study indicate that bovine CES1, CES2, CES3, CES5 and CES6 subunits have similar amino acid sequences with the corresponding human enzymes and share key conserved sequences and structures with human CES1. In addition, two bovine CES1 like subunits have charge clamp sequences in positions described for human CES1, which are consistent with oligomeric subunit structure for these enzymes. In contrast, bovine CES2 lacks the subunit-subunit binding residues reported for human CES1 and is consistent with the monomeric subunit structure reported for human and baboon CES2. This study also describes evidence for two CES1 'like' genes, which are located in tandem with the *CES5* gene, and with the more distantly located *CES2*, *CES3* and *CES6* genes on chromosome 18. Predicted secondary structures for bovine CES1, CES2, CES3, CES5 and CES6 showed a high degree of conservation with human CES1. Phylogeny studies indicated that the two *CES1* like genes have apparently appeared during mammalian evolution, well before (~ 60 MY ago) the appearance of the bovid common ancestor (~20 MY ago). In addition, bovine *CES2, CES3, CES5* and *CES6* genes were apparently generated from successive gene duplication events prior to the appearance of the eutherian and marsupial common ancestor, as described by Holmes and coworkers (2008a). Metabolic roles for the bovine CES isozymes remain to be determined, although it is likely that CES1.1, CES1.2 and CES2 are predominantly responsible for drug clearance (in liver), first pass metabolism of drugs (in intestine and lung) and lung surfactant metabolism, and may contribute to lipid and cholesterol metabolism in the body. The metabolic functions for the other bovine CES subunits await analyses of their differential tissue distributions and kinetic properties.

Acknowledgments

This project was supported by NIH Grants P01 HL028972 and P51 RR013986. In addition, this investigation was conducted in facilities constructed with support from Research Facilities Improvement Program Grant Numbers 1 C06 RR13556, 1 C06 RR15456, 1 C06 RR017515.

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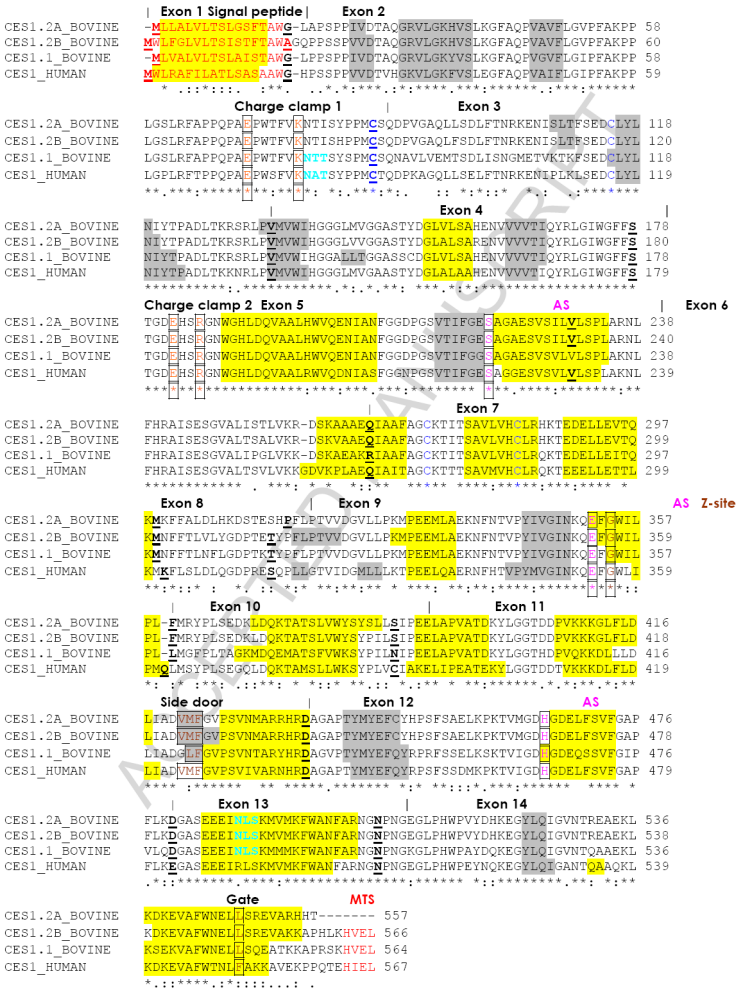

Figure 1. Amino acid sequence alignments for bovine and human CES1 subunits

See Table 1 for sources of CES sequences; * shows identical residues for human and bovine CES subunits; : 2 alternate residues. Residues involved in endoplasmic reticulum processing at N- (Signal peptide) and C- termini (MTS-microsomal (endoplasmic reticulum) targeting sequence); N-glycosylation residues at 79NAT (Human CES1) and potential N-glycosylation sites; ○ shows active site triad residues Ser, Glu, and His. 'Side door', 'Gate' residues and Cholesterol

binding Gly residue (E site) for human CES1 Disulfide bond ---- Cys residues for human CES1. Charge clamp residues identified for human CES1; Helix (Human CES1 or predicted helix; Sheet (Human CES1) or predicted sheet. Large font shows known or predicted exon junctions. Exons are numbered for human CES1. Residues in bold and underlined are predicted exon start sites.

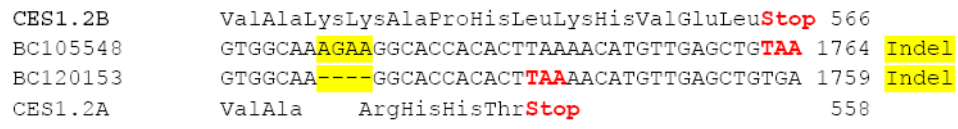

Figure 2. C-Terminal nucleotide and amino acid sequences for bovine CES1.2A and CES1.2B

GenBank Accession numbers are given for CES1.2A (BC120153) and CES1.2B (BC105548). Amino acid and nucleotide sequence numbers are shown. Stop refers to stop codon. Indel refers to deletion or insertion of 4 nucleotides.

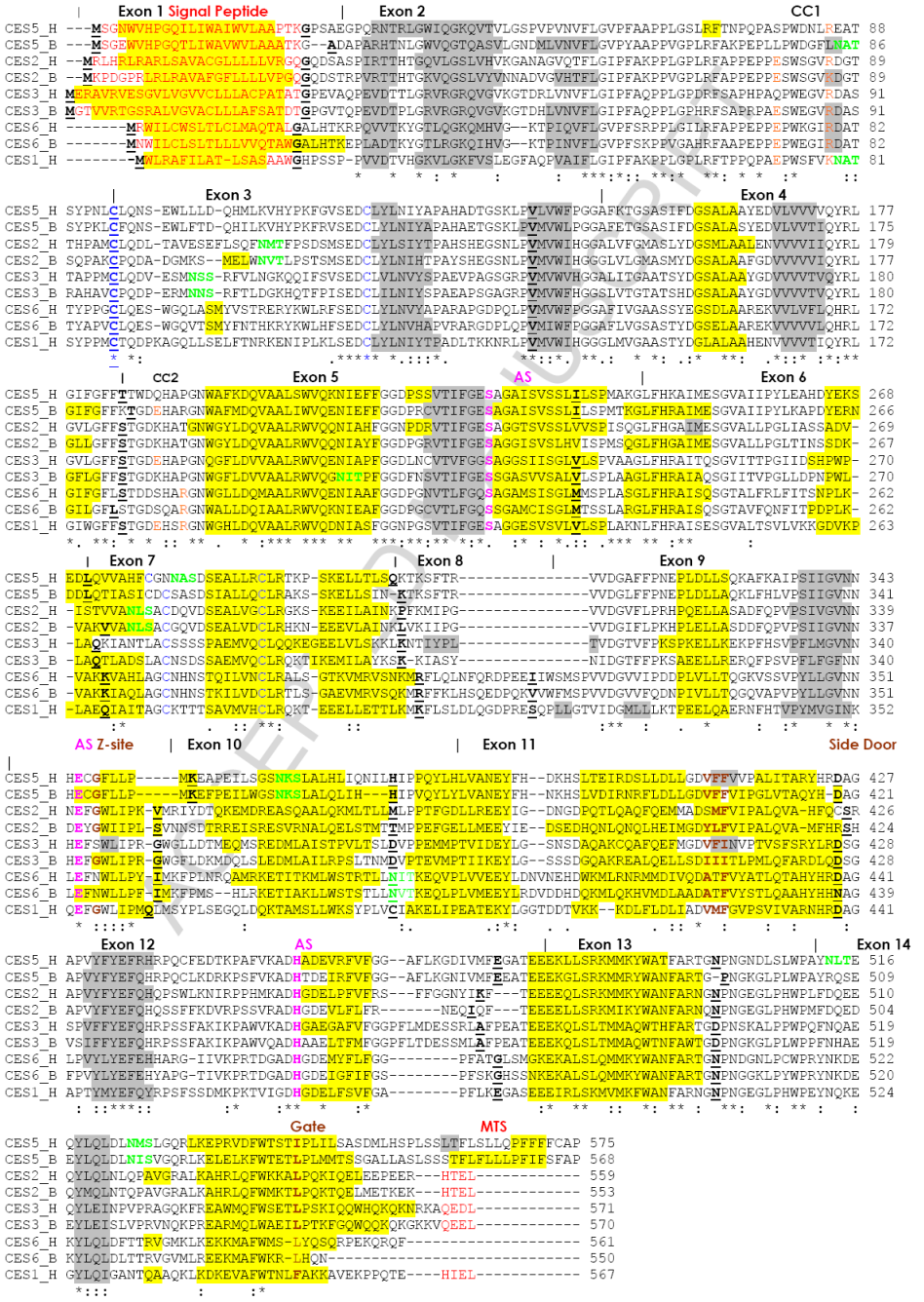

Figure 3. Amino acid sequence alignments for human CES1 and for human (H) and Bovine (B) CES2, CES3, CES5 and CES6 Subunits

See Table 1 for sources of CES sequences; * shows identical residues for CES subunits; : 2 alternate residues. Residues involved in endoplasmic reticulum processing at N- (Signal peptide) and C- termini (MTS-microsomal (endoplasmic reticulum) targeting sequence); N-glycosylation residues at 79NAT (Human CES1) and potential N-glycosylation sites; ▲ shows active site triad residues Ser, Glu, and His, 'Side door', Gate residues and Cholesterol binding Gly residue (E site) for human CES1; Disulfide bond ---- Cys residues for human CES1. Charge clamp residues identified for human CES1; Helix (Human CES1 or predicted helix; Sheet (Human CES1) or predicted sheet. Large font shows known or predicted exon junctions. Exons are numbered for human CES1. Residues in bold and underlined are predicted exon start sites.

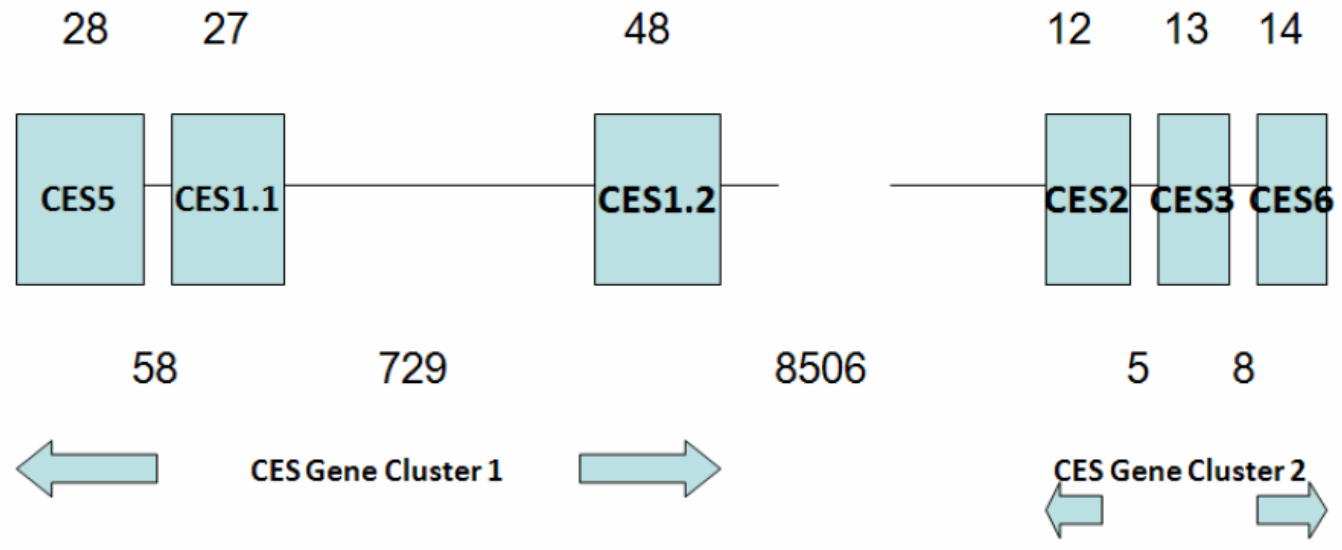

Figure 4. Predicted Locations of CES Genes on Bovine Chromosome 18
Numbers refer to kilobases of DNA.

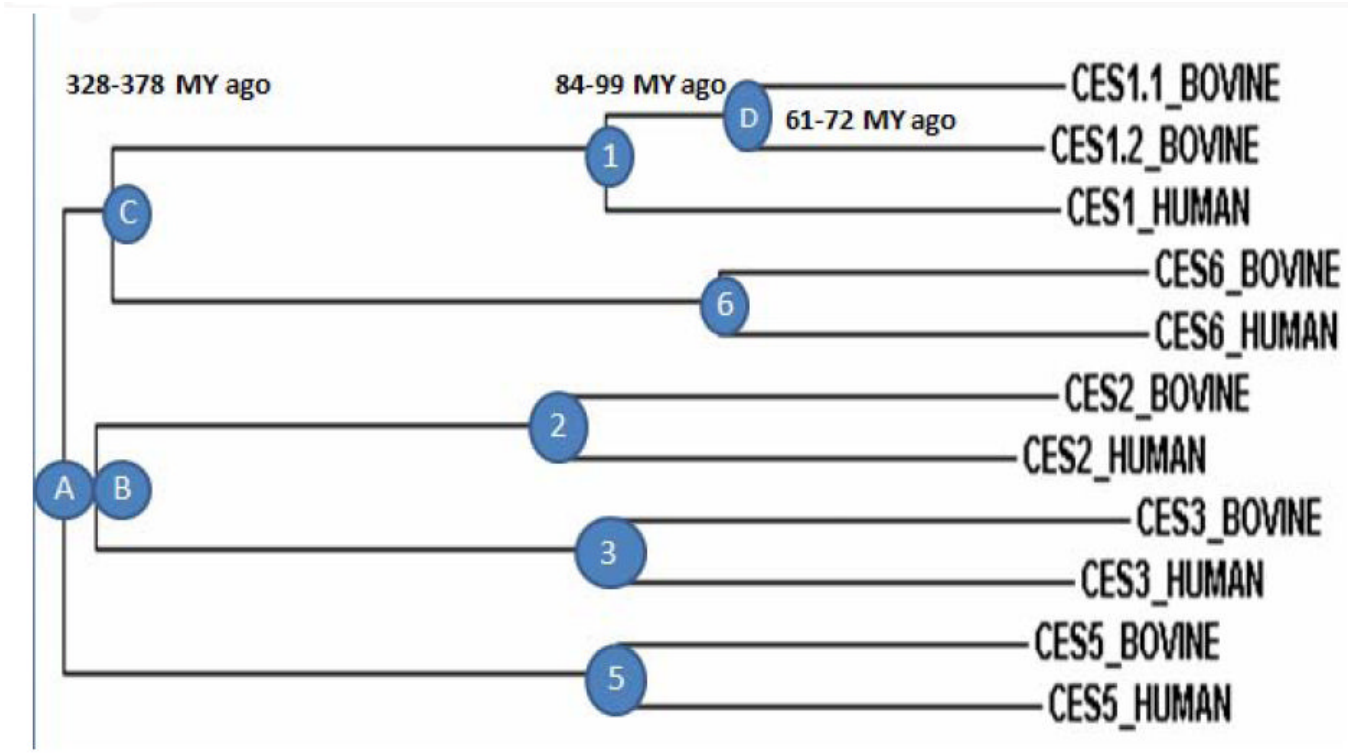

Figure 5. Phylogenetic Tree of Human and Bovine CES1, CES2, CES3, CES5 and CES6 Amino Acid Sequences

The tree is labeled with the gene name and the species name. Note the separation into 5 clusters for human and bovine CES1; CES2; CES3; CES5; and CES6. The gene duplication events generating the five distinct gene families (CES1, CES2, CES3, CES5 and CES6) have been previously estimated to have occurred ~ 328–378 million years ago (Holmes et al., 2008a). Numbers 1, 2 and 3 refer to predicted common ancestors for CES; CES2 and CES3; and CES1 and CES6, respectively. CA1, CA2, CA3, CA5 and CA6 refer to predicted common ancestors for eutherian mammalian CES1; CES2; CES3; CES5; and CES5, respectively. CA1c refers to a predicted common ancestor for bovine CES1.1 and CES1.2. Predicted dates for common ancestors are shown: 1–3: 328–378 MY (millions of years) ago; CA1–CA6: 84–99 MY ago; CA1c: 61–72 MY ago.

Table 1

Bovine and Human CES Genes and Protein Subunits

Mammal	CES Gene	GenBank ID ^ NCBI BLAST ID	* UNIPROT ID	No of Amino Acids	Chromosome Location	Strand	Exons	Gene Size (bps)	Alternate CES Gene Name
Bovine	CES1.1	**BC102781**	Q5MYB8	565	18:24,344,904-24,371,523	Negative	14	26620	BREH1
	CES1.2A	**BC120153**	QOVCI3	557	18:25,100,195-25,140,384	Negative	14	40190	CES
	CES1.2B	**BC105548**	Q2KJ30	566	18:25,100,195-25,148,621	Negative	14	48460	CES1 or EST1
	CES2	**BC10288**	Q3TOR6	553	18:33,654,184-33,665,882	Positive	12	11699	EST2
	CES3	^**XP590749**		570	18:33,671,230-33,683,956	Positive	13	12727	EST31
	CES5	^**XP591772**		576	18: 24,258,805-24,286,874	Positive	13	28070	CES7 or Cauxin
	CES6	**BC149217**	POC6R3	550	18:33,691,872-33,705,665	Positive	14	13794	ESTHL
Human	CES1	**L07765**	P23141	567	16:54,394,265-54,424,576	Negative	14	30311	EST1
	CES2	**Y09616**	O00348	559	16:65,525,828-65,536,493	Positive	12	10665	EST2
	CES3	**AY358609**	Q6UWW8	571	16:65,552,639-65,566,552	Positive	13	13913	EST3 or EST31
	CES5	**BC069501**	Q6NT32	575	16:54,437,867-54,466,634	Negative	13	28767	CES7 or Cauxin
	CES6	**AY358804**	Q5XG92	575	16: 65,580,177-65,600,543	Positive	14	20367	ESTHL

^ Derived following BLAST using human CES sequences and NCBI web tools

* UNIPROT ID of the CES protein using the SWISS-PROT Web Browser

Table 2

Percentage Identities for Bovine and Human CES Subunit Amino Acid Sequences

CES gene	hCES1	hCES2	hCES3	hCES6	hCES5	bCES1.1	bCES1.2	bCES2	bCES3	bCES6	bCES5
hCES1	100	45	42	43	42	71	76	43	41	41	42
hCES2	45	100	46	39	43	44	44	72	43	39	44
hCES3	42	46	100	40	41	42	41	43	71	40	43
hCES6	43	39	40	100	42	44	43	39	36	75	40
hCES5	42	43	41	42	100	43	42	44	41	42	76
bCES1.1	71	44	42	44	43	100	81	43	40	43	43
bCES1.2	76	44	41	43	42	81	100	43	40	46	42
bCES2	43	72	43	39	44	43	43	100	42	39	45
bCES3	41	43	71	36	41	40	40	42	100	39	41
bCES6	41	39	40	75	42	43	46	39	39	100	42
bCES5	42	44	43	40	76	43	42	45	41	42	100

Mammals: h-human; b-bovine

Table 3

Predicted N-Glycosylation Sites for Human and Bovine CES Subunits

Mammal	CES Gene	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9	Site 10	No of Sites
Human	CES1[,]	79NAT[,]										1
	CES2			111NMT	274NLS							2
	CES3		105NSS									1
	CES5					281NAS	363NKS			511NLT	522NMS	4
	CES6							377NIT				1
Cow	CES1.1	79NTT							491NLS			2
	CES1.2A								488NLS			1
	CES1.2B								490NLS			1
	CES2			109NVT	274NLS							2
	CES3		105NNS									1
	CES5	84NAT					361NKS			513NLT	517NIS	4
	CES6							375NVT				1

[,] Identified N-glycosylation site for human CES1 (Kroetz et al., 1993). Amino acid residues are shown: N-Asn; A-Ala; T-Thr; S-Ser; M-Met; L-Leu; I-Ile; K-Lys. The number refers to the first of three residues for the identified sites.

Table 4
Evolution of Human and Bovine CES Genes

CES Gene Common Ancestor[3]	Genetic Distance[5]	% Substitution Rate/My	Common Ancestor My Ago
CA1c	0.08	0.11-0.13	62-73[1]
CA1	0.109	0.11-0.13	84-99[2]
CA2	0.124	0.13-0.15	84-99[2]
CA3	0.141	0.14-0.17	84-99[2]
CA6	0.118	0.12-0.14	84-99[2]
CA7	0.118	0.12-0.14	84-99[2]
1	0.27±0.02[8]	0.07-0.08	328-378[4]
2	0.26[9]	0.07-0.08	325-371[6]
3	0.27[10]	0.07-0.08	328-378[7]

[1] Bovine CES1.1 and CES1.2 common ancestor;

[2] Bovine and Human CES1 common ancestor;

[3] CES gene common ancestor;

[4] Ancestral CES gene common ancestor;

[5] see methods;

[6] Common ancestor for ancestral CES2 and CES3 genes;

[7] Common ancestor for ancestral CES1 and CES6 genes;

[8] Average genetic distance (±standard error) for CES1, CES2, CES3, CES5 and CES6 genes;

[9] Average genetic distance for CES2 and CES3 genes;

[10] Average genetic distance for CES1 and CES6 genes.