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Elsevier Editorial System(tm) for Veterinary Immunology and Immunopathology
Manuscript Draft

Manuscript Number:

Title: Expressed gene sequences of two variants of sheep interleukin-25

Article Type: Technical Report

Keywords: sheep; cytokines; IL-25; IL-17E; transcript variants

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Expressed gene sequences of two variants of sheep interleukin-25

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Abstract

This report describes the cloning and characterization of sheep interleukin-25 (IL25) expressed gene sequences and shows that, like humans, sheep express two transcript variants of IL25. Transcript variant 1 (IL25v1) has a 510 bp open reading frame encoding a 169 amino acid polypeptide with a calculated M*r* 19,200. The 498 bp IL25v2 encodes a 165 amino acid polypeptide with a calculated M*r* 18,710; both with an isoelectric point equal to 8.0307. The additional 12 bp of IL-25 isoform 1 are at the 5' end and encode an MYQA peptide, otherwise their sequences are identical. Phylogenetic analysis shows that both sheep IL-25 isoforms are most closely related to cattle and pig IL-25.

Keywords: sheep, cytokines, IL-25, IL-17E, transcript variants.

The interleukin-17 (IL-17) family is a related group of proinflammatory 1 cytokines consisting of six members (IL-17A – F). They are structural homologues of the cysteine knot growth factors with a characteristic pattern of four cysteines forming the knot (Weaver et al., 2007).

IL-17A and F are produced by Th17 cells and act by stimulating IL-1β, IL-6, IL-8 and TNFα;

IL-17B, C and D have similar, but subtly different functions and originate from non-lymphoid sources (Li et al., 2000). The differences in their functions seem to be associated with the complexities of homo- or heterodimerization and differential interactions with the three receptor complexes (IL17RA – C) (Weaver et al., 2007). However, treatment of mice with these cytokines results in a consistent phenotype characterized by acute inflammation associated with neutrophil infiltration (Shi et al., 2000; Hurst et al., 2002; Fouser et al., 2008). Of particular interest is the role of IL-17A in the induction of IL-12 and IFNγ and the promotion of the Th1 response (Lin et al., 2009).

In contrast IL-25 (IL-17E) is expressed largely by CD4+ Th2 cells. Interaction of IL-25 with its receptor complex (EV127 or IL17RB) on 'nuocytes', cells that lack classical B, T and macrophage markers (Lee et al., 2001; Neill et al., 2010), potentiates the expression of NFATc1 and jun-B (Angkasekwinai et al., 2007) and promotes Th2 responses through the induction of GATA3 and the expression of IL-4, IL-5 and IL-13 (Weaver et al., 2007). This results in IL-25 being a critical link between innate and adaptive immunity and it is now recognized as a key component of protection to gastrointestinal helminths (Weaver et al., 2007) as well as Th2-associated pathologies through IL-5 driven eosinophilia (Fort et al., 2001). Our interest in sheep IL-25 originates from work concerned with the immunological and genetic basis of resistance to the gastrointestinal parasitic nematode of sheep, *Teladorsagia circumcincta* (Beraldi et al., 2008).

Total RNA was isolated from sheep gastric lymph nodes using a RiboPure™ Kit

(Ambion, Huntingdon, UK) according to the manufacturer's instruction. RNA quality and quantity were determined with an Agilent Bioanalyzer using RNA 6000 Nano Kit (Agilent, South Queensferry, UK) and NanoDrop ND-1000 (Thermo Fisher Scientific, Loughborough, UK). cDNA was synthesized from total RNA with Superscript III reverse transcriptase using an oligo-dT primer (Invitrogen, Paisley, UK). RT-PCR was performed using primers based on the bovine IL25 sequence (GenBank acc # XM_605190.2) to amplify a partial cDNA sequence of sheep IL25; forward primer 5'- GAGGAGTGGCTGAAGTGGAAC-3' and reverse primer 5′-CGGTAGAAGACGGTCTGGTTG-3'. RT-PCR was performed using FastStart Taq DNA Polymerase (Roche Diagnostics Ltd., Lewes, UK): 95 °C for 4 min, 35 cycles of 95 °C for 30 s, 30 s at 59 °C, 72 °C for 60 s and a final elongation step of 72 °C for 7 min. The amplified fragments were purified and cloned into pGEM-T Easy vector (Promega, Southampton, UK) as per the manufacturer's instruction. A random selection of resultant clones were then sequenced on both strands using T7 and SP6 sequencing primers in separate reactions with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). ABI 3730 capillary sequencing of samples was performed at GenePool (http://genepool.bio.ed.ac.uk/).

To obtain the 5' transcript sequence for sheep IL25, a gene specific primer (GSP) was designed based on the partial sheep IL25 sequence obtained as previously described. Using 5′-RACE, total RNA was reverse transcribed with Transcriptor Reverse Transcriptase (5'/3' RACE kit, 2nd Generation, Roche) using the sheep IL25 GSP reverse primer (5′-CGGTCTGGTTGTGGTAGAG-3′) to obtain single stranded cDNA. This was purified before the addition of a homopolymeric A-tail to the 3' end of the first strand cDNA using terminal transferase and dATP (5'/3' RACE kit). PCR was carried out using a Oligo dT11

Anchor primer (supplied in 5'/3' RACE kit) as the forward primer and a nested reverse second

GSP primer (5′-CGGTCCAAGTCTCTGTCCAA-3') using FastStart Taq DNA Polymerase: 95 °C for 4 min, 10 cycles of 95 °C for 15 s, 30 s at 55 °C and 72 °C for 40 s, then 25 cycles of 95 °C for 15 s, 30 s at 50 °C and 72 °C for 40 s (increasing by 20 s per cycle) and a final elongation step at 72 °C for 7 min. A second round of PCR amplification was performed using a nested third GSP (5'-ATGGAACGGCTGTTGAGG -3') and the PCR Anchor primer (5'/3' RACE kit) using FastStart Taq DNA Polymerase with the following conditions: 95 °C for 4 min followed by 30 cycles of 95 °C for 30 s, 30 s at 63 °C and 72 °C for 60 s and 72 °C for 7 min. After separation of the amplified products by agarose gel electrophoresis the bands were excised and the DNA extracted using a QIAquick Gel Extraction Kit (Qiagen, Crawley, UK). Purified PCR fragments were cloned into pGEM-T Easy (Promega) and the resultant clones were sequenced on both strands as previously described.

The 3' transcript sequence for sheep IL25 was obtained using a forward primer based on the partial IL25 sequence and a reverse primer based on the bovine genome sequence in the 3' UTR region of IL25 (Btau_4.2;Chr10; scaffold25; NW_001492802, 387124-44). Total RNA was reverse transcribed with Superscript III reverse transcriptase using an oligo-dT primer (Invitrogen) to obtain first strand cDNA. PCR was then carried out using a sheep IL25 forward primer (5′-TGTCCACACTGTGTCAGC-3′) and reverse primer (5'-TGGTTGTCCAACCAGCTCCAG-3') using FastStart Taq DNA Polymerase: 95 °C for 6 min, 35 cycles of 95 °C for 30 s, 30 s at 60 °C and 72 °C for 45 s, and a final elongation step at 72 °C for 7 min. Amplified products were purified and cloned into pCR2.1 vector using the TOPO® TA Cloning® Kit (Invitrogen). A random selection of resultant clones were then sequenced on both strands using M13 forward and reverse primers in separate reactions as described previously with the sequence files compiled using CLC Sequence Viewer v6.4 (www.clcbio.com).

The cDNA sequences of two transcript variants of sheep IL25 are shown in Fig.1 (Genbank acc # sheep IL25v1, FR670343; sheep IL25v2, FR670344). This shows that the two variants have identical coding sequences except that IL25v1 has an additional 12 bp sequence (ATGTACCAGGCG) at the 5' end. These sequences map to OAR7:22,059,878-22,064,877; Ovine Genome Assembly v1.0 (http://www.livestockgenomics.csiro.au/sheep/oar1.0.php).

The amino acid sequences (Genbank acc # sheep IL-25 isoform 1 CBW31643.1; sheep IL-12 isoform 2 CBW31644) were deduced using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). A 510 bp open reading frame of sheep IL25v1 is predicted to encode a 169 amino acid polypeptide (IL-25 isoform 1) with a calculated Mr 19,200. The 498 bp sheep IL25v2 is predicted to encode a shorter polypeptide (IL-25 isoform 2) of 165 amino acids with a calculated Mr 18,710; both isoforms have an isoelectric point equal to 8.0307. The signal peptide cleavage sites were predicted using SignalP 3.0 Server (Emanuelsson et al., 2007); the SignalP-NN and SignalP-HMM algorithms predicted different cleavage sites for IL-25 isoform 1, between residues 16-17 (THT-LC) and between residues 20-21 (CLG-SQ). The only cleavage site predicted for IL-25 isoform 2 was CLG-SQ, between residues 16-17. The predicted cleavage site for both human isoforms is THT-YS (isoform 1, 32-33 and isoform 2, 16-17); which is equivalent to the THT-LC site (residues 16-17) in sheep IL-25 isoform 1. In relation to the other mammalian IL-25 sequences both human sequences have an 8 amino acid deletion (LCLGSQRR) between residues, which contains the CLG-SQ putative sheep cleavage site.

The predicted amino acid sequences of other mammalian IL-25 proteins were aligned (Fig. 2) using ClustalW2 (Larkin et al., 2007). Quantitative sequence alignment was performed using Emboss::needle (http://www.ebi.ac.uk/Tools/emboss/align/). The sheep IL-25 isoform 1 sequence shares 81% identity with the cattle amino acid sequence (Table 1). This relatively low level of identity between these two closely-related species is largely due to cattle IL-25

having an additional MNQNLREQLGEGASRLGRSSFLTSL peptide at the NH2 terminus. Sheep IL-25 isoform 1 shares 64% and 67% identity with the pig and human isoform 1 amino acid sequences respectively largely due to the fact that pig IL-25 has a 48 residue NH2-terminal extension and human isoform 1 has a 16 residue NH2-terminal extension in relation to sheep IL-25 isoform 1. The relatively high level of sequence identity between sheep IL-25 isoform 1 and horse, dog, human isoform 2, rat and mouse is largely because they all have the same start site. The levels of identity between sheep IL-25 isoform 2 and the other mammalian IL-25 sequences are slightly lower than sheep IL-25 isoform 1 because it is shorter by 4 amino acids at its NH2 terminus.

The phylogenetic relationship of mammalian IL-25 was investigated using Cobalt (http://www.ncbi.nlm.nih.gov/tools/cobalt/) and CLC Sequence Viewer v6.3. The resulting phylogram (Fig. 3) clearly shows that the two sheep IL-25 isoforms are phylogenetically closest to cattle and pig IL-25 despite the relatively low level of sequence identity; and are

relatively distant to horse and dog IL-25 despite a relatively 1 high level of sequence identity. This is largely because of variations at the NH2 terminus of mammalian IL-25 proteins. Analysis of the biological role of IL-25 in inflammation and immunity at mucosal surfaces requires the facility to identify and measure cytokine levels in cells and tissues. Antibody reagents are not yet available for many sheep cytokines and consequently we have developed quantitative real-time RT-PCR (RT-qPCR) for sheep IL25. First strand cDNA synthesis was performed with 0.5 μg of gastric lymph node total RNA using 50 μM oligo (dT)20, 40 U RNaseOUT, 200 U Superscript ™ III reverse transcriptase (RT), 5× RT buffer, 25 mM MgCl2 and 0.1 M DTT (Invitrogen). Using the sequence FR670343, forward 365 – 383 (TGGCTGAAGTGGAACAGTG) and reverse 544 – 562 (GACACAGTGTGGACACAGG) primers (Invitrogen) were selected to amplify a 198 nt amplicon, which was verified by DNA sequence analysis.

RT-qPCR was performed using the Rotor-Gene 3000 (Qiagen) and SYBR Green I detection and reactions were prepared using a CAS-1200™ Precision Liquid Handling System (Qiagen). All reactions were in a 10 µl final volume containing 5 µl of cDNA at the optimum dilution (1:10), 0.75 U FastStart Taq DNA Polymerase, 10× PCR reaction buffer, MgCl2 at optimum concentration (2.5 mM), 200 µM of each dNTP (Roche), 0.35 µl SYBR Green I (diluted 1/1000 in DMSO) and 0.45µl of each primer (final concentration 450 nM). The amplification profile was 5 min at 94ºC, followed by 40 cycles of 10 s at 94ºC, 10 s at 62ºC, 15 s at 72ºC, and followed by a dissociation curve analysis program to confirm a single gene product. The quantitative data were analysed using the Rotor-Gene 3000 Software version 6.1.93 (Qiagen).The dynamic range of the standard curve spanned Cq 9.89 – 24.05 and was used to derive the copy number of the target sequence in unknown samples. The reaction efficiency of this optimized assay was 101%, with R2 24 of 0.99 and slope of -3.258.

Acknowledgments

VV is a The Ford Foundation (http://www.fordfound.org/) International Fellow.

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Figure Legends

Fig. 1. Nucleotide sequence of coding regions of sheep IL25 cDNA transcript variant 1 (FR670343) and transcript variant 2 (FR670344)

Fig 2. Alignment of sheep IL-25 deduced amino acid sequences (IL-25 isoform 1, CBW31643.1 and IL-25 isoform 2, CBW31644.1) with cattle (XP_605190.2), pig (XP_001926321.1), dog (XP_537375.2), horse (XP_001918360.1), human variant 1 (NP_073626.1) and variant 2 (NP_758525.1), rat (XP_001054877.1) and mouse (NP_542767.1) sequences.

Fig. 3. Phylogram showing the relationship of sheep IL-25 isoform 1 and IL-25 isoform 2 sequences and other mammalian IL-25 sequences; based on the deduced amino acid sequences.

Tab.1

Sequence identity[a] of IL25 nucleotide and deduced amino acid sequences

	sh v1	*sh v2*	*cattle*	*horse*	*pig*	*dog*	*hum v1*	*hum v2*	*rat*
sheep v2	**98** *98*								
cattle	**84** *81*	**82** *80*							
horse	**88** *82*	**86** *80*	**76** *71*						
pig	**67** *64*	**65** *63*	**75** *69*	**65** *62*					
dog	**84** *78*	**82** *77*	**73** *68*	**85** *81*	**65** *60*				
human v1	**74** *67*	**72** *66*	**77** *70*	**77** *73*	**67** *62*	**72** *66*			
human v2	**81** *74*	**79** *72*	**71** *65*	**85** *82*	**61** *59*	**79** *73*	**90** *90*		
rat	**80** *75*	**78** *73*	**70** *64*	**82** *76*	**60** *57*	**79** *72*	**72** *69*	**79** *76*	
mouse	**82** *76*	**79** *73*	**71** *65*	**82** *78*	**61** *57*	**79** *72*	**74** *69*	**82** *76*	**91** *91*

[a] calculated using Needle (global); www.ebi.ac.uk/Tools/emboss/align/

Bold – nucleotide; italics – amino acid.

Comparison with cattle (Bos taurus # XM_605190.2), horse (Equine caballus # XM_001918325.1), pig (Sus scrofa # XM_001926286.1), dog (Canis familiaris # XM_537375.2), human (Homo sapiens v1 # NM_022789.2 and Homo sapiens v2 # NM_172314.1), rat (Rattus norvegicus # XM_001054877.1) and mouse (Mus musculus # NM_080729.2) IL25 sequences.

1 ATGTACCAGG CG**ATG**GCGTT CTTGGCAGTG GTCATGGGAA CCCACACCCT CTGTTTGGGG 60

61 TCCCAGAGGC GTTGCACCCA CTGGCCCGGC TGCTGCCCCA GCGAAGGACA GAACCCCACT 120

121 GAGGAGTGGC TGAAGTGGAA CAGTGTGCTC ATGCCTCCCC CAGAGACCAC CAGCCTCGCC 180

181 CACCACTCAG AATCCTGCAG TTCCAGCAAG GATGGACCCC TCAACAGCCG TTCCATCGCC 240

241 CCCTGGAGAT ATGAGTTGGA CAGAGACTTG AACCGGCTCC CGCAGGATCT GTACCACGCA 300

301 CGCTGCCTGT GTCCACACTG TGTCAGCCTC CAGACGGGCT CCCACATGGA CCCCCTGGGA 360

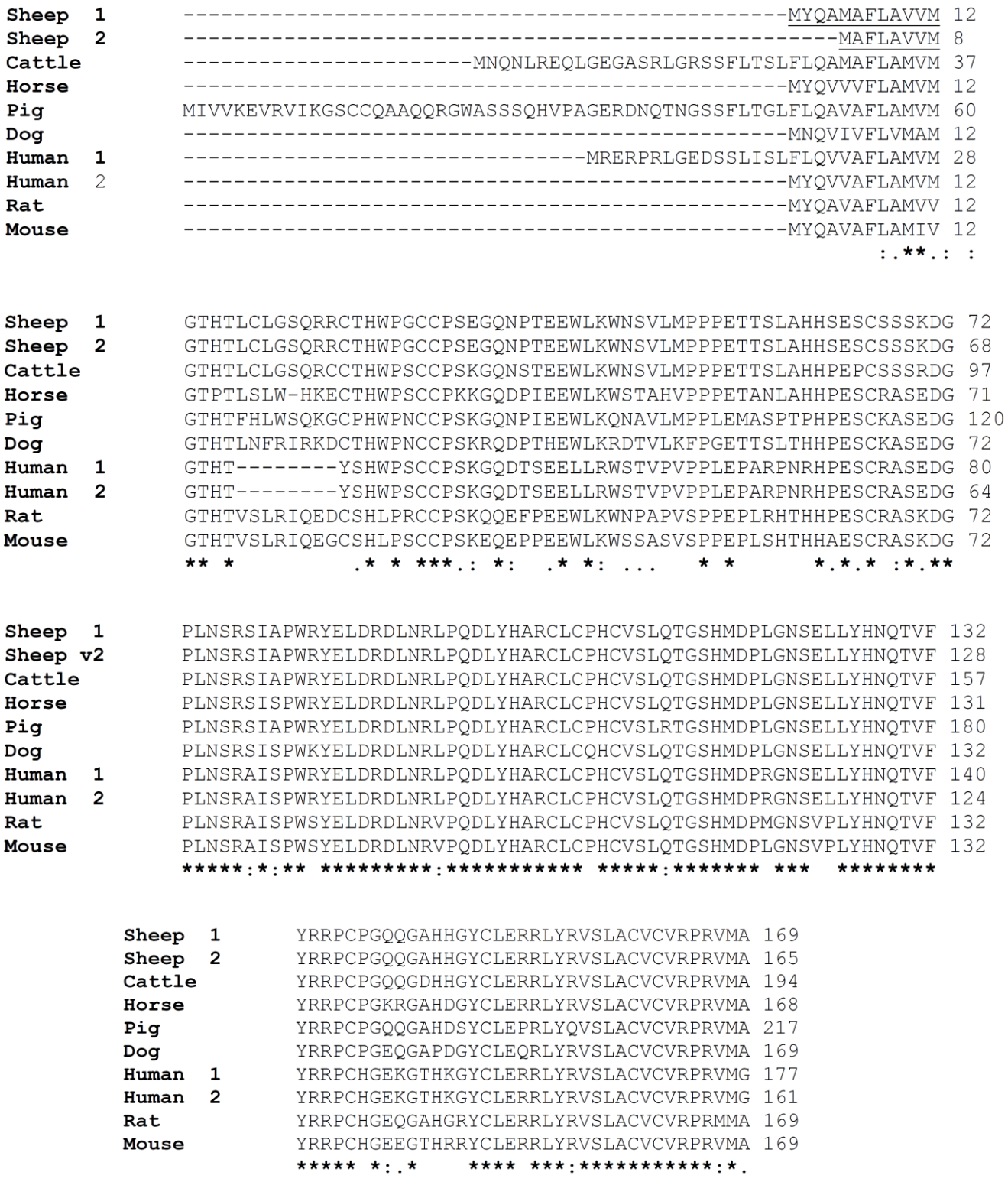
361 AACTCAGAGC TGCTCTACCA CAACCAGACC GTTTTCTACC GGCGGCCATG CCCTGGACAG 420

421 CAGGGCGCCC ACCATGGCTA CTGCCTGGAA CGCAGGCTCT ACCGTGTCTC CTTGGCTTGC 480

481 GTGTGCGTGC GGCCCCGTGT GATGGCCTAG 510

Underlined ATG is the first codon of transcript variant 1 (IL25v1); bold ATG is the first codon of transcript variant 2 (IL25v2)

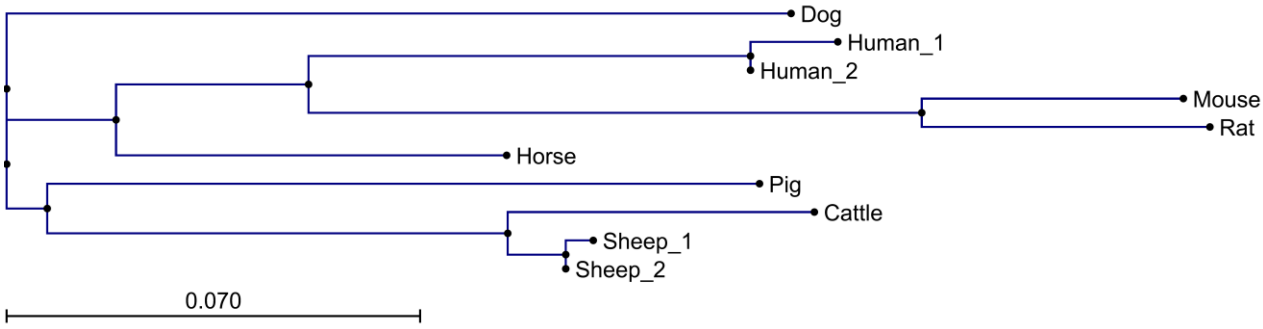
Figure



alignments calculated using ClustalW2.

* nucleotides are identical in all sequences **:** conserved substitutions **.** semi-conserved substitutions

Figure


Dog
Human_1
Human_2
Mouse
Rat
Horse
Pig
Cattle
Sheep_1
Sheep_2
0.070