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The bovine aristaless-like homeobox 4 *(ALX4)* as a candidate gene for syndactyly

A. Wöhlke[a] H. Kuiper[a] O. Distl[a] C. Drögemüller[a, b]

[a]Institute of Animal Breeding and Genetics, University of Veterinary Medicine Hannover, Hannover (Germany)
[b]Institute of Genetics, Vetsuisse, University of Berne, Berne (Switzerland)

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Abstract. The *ALX4* (aristaless-like homeobox 4) gene encodes a paired-type homeodomain transcriptional activator and plays a major role in anterior-posterior pattern formation during limb development. Here, the cloning, genomic structure and expression of the bovine ortholog of the *ALX4* gene are reported. The bovine *ALX4* gene consists of four exons and is located on BTA15q28→q29 in a region syntenic to HSA11p11.2. The transcribed *ALX4* mRNA encodes a 397-amino-acid protein showing a paired-type homeodomain and a C-terminal stretch of amino acids known as the OAR- or aristaless domain. The predicted protein shares 92.5% identity to human and mouse ALX4 proteins and all three species share almost complete identity in the conserved domains. *ALX4* expression was detected by reverse transcriptase polymerase chain reaction in bovine fetal limb bones. The *ALX4* gene was evaluated as a candidate gene for bovine syndactyly which has been mapped on the telomeric region of cattle chromosome 15. Sequencing of the four exons with flanking sequences of the bovine *ALX4* gene from a panel of 14 affected animals belonging to German Holstein, German Fleckvieh and crossbreds, and 27 unaffected individuals from German Holstein revealed five silent SNPs within the coding region out of eleven SNPs in total. Four SNPs were polymorphic in the affected animals, but in comparison to the genotyped unaffected individuals the genotype distribution showed no evidence for an association to the phenotype. Therefore our data indicate that the *ALX4* gene can probably be excluded as candidate gene for bovine syndactyly in the examined animals.

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Syndactyly ('mulefoot') in cattle is caused by a fusion or non-division of the two digits of the bovine foot (Leipold et al., 1973). This bovine congenital anomaly follows a monogenic recessive inheritance with incomplete penetrance and variable expressivity (Nicholas, 2003). The forelegs are more frequently affected, but also the hind legs or all four feet can be involved. Syndactyly could be observed in many cattle breeds (Krölling, 1956; König et al., 1980; Leipold et al., 1998) but most of the literature concerns the incidence in Holstein cattle (Eldrige et al., 1951; Leipold et al., 1969; Ojo et al., 1975; Charlier et al., 1996; Bähr et al., 2004). A whole genome linkage study based on affected Dutch Holstein cattle showed that the responsible recessive gene for syndactyly is located on the telomeric end of cattle (BTA) chromosome 15 (Charlier et al., 1996). This chromosomal location was confirmed by another linkage analysis in a pedigree of affected German Holstein cattle (Drögemüller and Distl, 2006). According to the cattle–human comparative map this chromosomal region is predicted to contain genes of human (HSA) chromosome 11p (Gautier et al., 2002). We hypothesized that a mutation in the *ALX4* (aristaless-like homeobox 4) gene located on HSA11p11.2 (NCBI map viewer, human genome build 35.1) could be responsible for bovine syndactyly because of its role in mammalian bone development. The *ALX4* gene encodes a paired-type homeo-

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Request reprints from Dr. Cord Drögemüller
Institute of Genetics, University of Berne
Bremgartenstrasse 109a, 3001 Berne (Switzerland)
telephone: +41 31 631 2529; fax: +41 31 631 2640
e-mail: cord.droegemueller@itz.unibe.ch

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Table 1. PCR primers for the amplification of bovine *ALX4* exons

Exon	Primer	Sequence (5′ → 3′)	Product length (bp)	Annealing temperature (°C)
1	F	GCA TGA ATG CTG AGA CTT GC		
	R	AGC AAG TTG ATC GCG TTT C	500	60
2	F	TCC CTC CTA CCT CTC GGG C		
	R	CCT GTC TCG GGC CAC TG	442	60
3	F	GAT TCT GCC GTA GTC TGT GG		
	R	CTT CAG CAT TCC TCG GTT C	786	58
4	F	AAA GCC TCC CAG GTA AAC AC		
	R	GAA AGT GCT GAG GGT CAG G	611	58

domain transcriptional activator related to *Drosophila* aristaless and plays its major role in anterior-posterior pattern formation during limb development (Niswander, 2003). A series of mouse mutants has been reported to exhibit preaxial polydactyly because of ectopic sonic hedgehog (Shh) expression. One such mutant, Strong's luxoid polydactyly, is caused by loss of function of *Alx4* (Qu et al., 1998; Takahashi et al., 1998). Alx4 may negatively regulate Shh expression in the anterior margin of the developing limb bud. Hence, loss of its function results in ectopic Shh expression and the associated preaxial polydactyly. In human cases of inherited parietal foramina (PFM) phenotype (OMIM 168500) characterized by ossification defects in the parietal bones are caused by *ALX4* mutations (Wuyts et al., 2000). Because of the absence of limb defects in humans with PFM, it appears that the pattern formation function during limb development is not as sensitive to dosage as is its proliferative/cell survival function during cranial suture development. According to previous results in mice (Qu et al., 1997), human *ALX4* expression appears to be restricted to bone (Wu et al., 2000).

Here we characterize the bovine *ALX4* gene, and evaluate this gene as a positional and functional candidate gene for syndactyly in cattle.

Materials and methods

Cloning of the bovine ALX4 gene

For the isolation of a bovine bacterial artificial chromosome (BAC) clone containing the *ALX4* gene the National Center for Biotechnology Information (NCBI) genome survey sequence database division was searched with 135 kb human genome sequence containing the human *ALX4* gene (GenBank acc. no. NT_009237.17; Hs11_9394:43076277-43211131) using the basic local alignment search tool nucleotide (BLASTN; http://www.ncbi.nlm.nih.gov/BLAST/). With this database search two bovine BAC end sequence reads of a single BAC clone (CH240-78B8) could be isolated with an expect value (E value) of 7e-31 (BAC end sequences GenBank acc. nos. BZ928142, BZ928075). The insert size of 215 kb of the BAC clone was determined by pulsed field gel electrophoresis. To confirm the presence of the bovine *ALX4* gene on the BAC clone polymerase chain reaction (PCR) with the primer pairs for the amplification of exon 1 to 4 (Table 1) was performed using DNA of CH240-78B8. The human exostoses (multiple) 2 *(EXT2)* gene is located 19,178 bp upstream of the *ALX4* gene on HSA11p12-p11 (NCBI map viewer, human genome build 35.1). Using a primer pair located in exon 16 of the bovine *EXT2* gene (see below) the presence of *EXT2* in the BAC clone could be confirmed as well.

Mapping of the bovine ALX4 gene

Two primer pairs, one from the bovine BAC Sp6 end sequence (CH240-78B8-Sp-F TTG GAA AGA GCC AAG AGC AG, CH240-78B8-Sp-R CAT AAT GGG GAG CTC TCA GG) and another one created from exon 16 of the bovine *EXT2* gene (mRNA GenBank acc. no. NM_177496; EXT2-F TGA AGA GCT TCC CCA ACA TC, EXT2-R AGG CAT CAA ACG CCA CTA AG) were used for the analysis of the Roslin/Cambridge bovine radiation hybrid (RH) panel purchased from Research Genetics (Huntsville, Ala., USA). The RH panel was created by exposing the bovine cell line to 3,000 rad of X-rays and fusion with non-irradiated HPRT hamster recipient cells (Wg3H) (Williams et al., 2002). The panel consists of 94 clones, complemented by a bovine and hamster genomic DNA as a control. Two independent PCR reactions were performed in a total of 12 μl using 25 ng of RH cell line DNA. PCR products were separated on 1% agarose gels. Scoring of PCR products was carried out independently by two investigators. Two-point linkage RH map calculations were carried out using the RHMAP3.0 package (Lange et al., 1995). Additionally two previously mapped BTA15 microsatellite markers (BMS820 and RM004; Gautier et al., 2002) were typed on the RH panel as well.

The 215-kb bovine BAC clone CH240-78B8 containing the bovine *ALX4* and *EXT2* genes was labeled with digoxigenin by nick translation using a Nick-Translation-Mix (Roche, Mannheim, Germany). Fluorescence in situ hybridization (FISH) on GTG-banded bovine chromosomes (ISCNDB 2000) was performed using 750 ng of digoxigenin-labeled BAC DNA. One μg sheared total bovine DNA and 10 μg salmon sperm were used as competitors in this experiment. After hybridization overnight, signal detection was performed using a Digoxigenin-FITC Detection Kit (Quantum Appligene, Heidelberg, Germany). The chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide and embedded in antifade. Thirty metaphases that were previously photographed were re-examined after hybridization with a Zeiss Axioplan 2 microscope equipped for fluorescence.

The bovine ALX4 cDNA and DNA sequences

In order to get sequences for the bovine *ALX4* gene we used the corresponding human and mouse mRNA (human acc. no. NM_021926; mouse acc. no. NM_007442) as query for BLAST searches against the NCBI Trace archive. With help of these two sequences we were able to isolate bovine genomic sequences for all four *ALX4* exons with parts of the introns. To assure the bovine *ALX4* gene structure primers for full length cDNA amplification were designed from homologous bovine sequences of the putative first and last exon, respectively. A forward primer was positioned in the sequence identical to *ALX4* exon 1 (GenBank acc. no. TI 118679805; ALX4-F1 TGC TGA GAC TTG CGT CTC TTA C) and a reverse primer in the sequence identical to *ALX4* exon 4 (GenBank acc. no. TI 86758262; ALX4-R3 CAT GTG GCC CAG GAG ATG). The experimentally derived bovine cDNA was used in BLAST searches against the current cattle genome assembly (build 2.1; http://www.ncbi.nlm.nih.gov/genome/seq/BtaBlast.html). For the exact localization of the exon/intron boundaries, the mRNA-to-genomic alignment program Spidey (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/index.html) was used. The GC content was cal-

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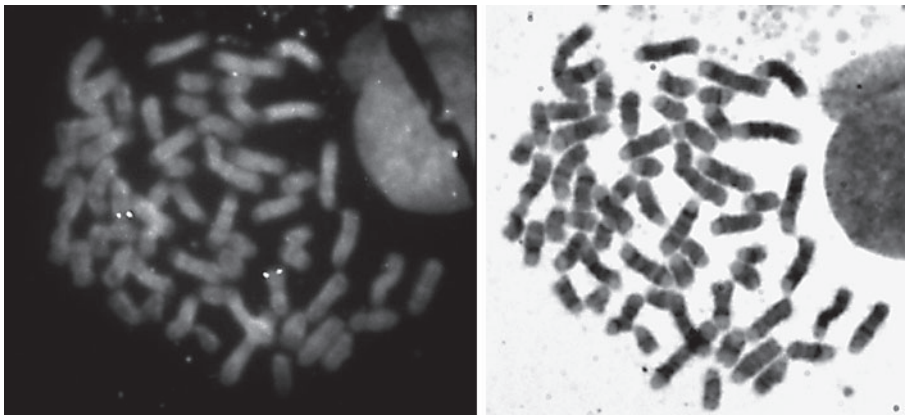

Fig. 1. Chromosomal assignment of the bovine *ALX4* gene by FISH analysis. The digoxigenin-labeled BAC clone CH240-78B8 containing the bovine *ALX4* gene was hybridized to GTG-banded metaphase chromosomes of a normal cattle. Double signals are visible on both chromosomes 15q28-q29. The chromosomes were counterstained with propidium iodide and subsequently identified by DAPI staining.

culated with the EBI toolbox CpG Plot/CpGreport (http://www.ebi.ac.uk/Tools/sequence.html).

For cDNA amplification total RNA from humerus and femur of a 2-month-old bovine fetus and a stillborn 9-month-old calf were isolated using the RNeasy 96 universal tissue kit (Qiagen, Hilden, Germany). Additionally we isolated RNA of lung and liver from the bovine fetuses. Aliquots of 1 μg total RNA were reverse transcribed into cDNA using 20 pmol $(T)_{24}V$ primer and Omniscript™ Reverse Transcriptase (Qiagen) in 20-μl reactions. One μl of the cDNA was used as template in a reverse transcriptase polymerase chain reaction (RT-PCR) reaction. The reaction was performed in a total of 25 μl containing 100 μM dNTPs, 25 pmol of each primer, the reaction buffer supplied by the manufacturer (Qiagen), and 1 U Taq polymerase. After a 5-min initial denaturation at 94°C, 35 cycles of 30 s at 94°C, 1 min at 58°C, and 45 s at 72°C were performed in an MJ Research thermocycler (Biozym, Hess. Oldendorf, Germany). The PCR products were directly sequenced with the DYEnamic ET Terminator kit (Amersham Biosciences, Freiburg, Germany) and a MegaBACE 1000 capillary sequencer (Amersham Biosciences).

Mutation analysis

For the identification of variations within the bovine *ALX4* gene the four exons with flanking regions were PCR amplified. The PCR was performed with genomic DNA of eight syndactylous calves and 27 samples from unaffected dams, sires, and half-sibs from a single German Holstein pedigree segregating for syndactyly (Drögemüller and Distl, 2006). Additionally, three single unrelated affected German Holstein calves, a single affected German Fleckvieh calf and two unrelated crossbred calves showing syndactyly were evaluated for possible causative *ALX4* polymorphisms.

PCR primers were generated with the primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are given in Table 1. The PCR was performed in 50-μl reactions containing 25 ng genomic DNA, 100 μM dNTPs, 50 pmol of each primer, and 2 U Taq polymerase in the reaction buffer supplied by the manufacturer (Qiagen). After 5 min initial denaturation at 94°C, 35 cycles of 30 s at 94°C, 30 s annealing temperature, and 80 s at 72°C were performed in an MJ Research thermocycler (Biozym). The PCR products were directly sequenced as described above.

Results and discussion

Mapping of the bovine ALX4 gene

PCR amplification of bovine *ALX4* and *EXT2* sequences on BAC CH240-78B8 DNA confirmed the presence of these genes on the BAC clone. Therefore this BAC was used as probe for FISH mapping on bovine metaphase chromosomes. The physical assignment localized the BAC CH240-78B8 to BTA15q28→q29 (Fig. 1). This chromosomal assignment is consistent with established syntenies between human chromosome 11 and bovine chromosome 15 (Amarante et al., 2000; Gautier et al., 2002; Snelling et al., 2004). To refine the localization of this BAC on BTA15 the Roslin/Cambridge bovine radiation hybrid panel was analyzed using two independent primer pairs revealing a unique signal pattern. Two-point analysis revealed close linkage of the BAC clone containing the *ALX4* and *EXT2* gene to the BTA15 microsatellite markers BMS820 and RM004 at a distance of 6.2 cR (LOD score 17.7) and 30.9 cR (LOD score 10.0), respectively. The available sequences of BMS820 and RM004 were subjected to BLAST comparisons against the sequence of the cow genome (build 2.1). A significant and unique match (E value $<10^{-5}$) against cattle genomic sequences was observed for BMS820 on chromosome 15 contig NW_928967 about 950 kb upstream to *ALX4* exon 1 (see below). The obtained in silico match confirmed the experimentally derived mapping result. Therefore the bovine *ALX4* map position fits to the previously established critical syndactyly gene region in cattle (Charlier et al., 1996; Drögemüller and Distl, 2006).

Sequence analysis of the bovine ALX4 gene

Due to the fact that no bovine *ALX4* EST was available the cDNA sequence for all four *ALX4* exons was derived experimentally. We obtained 1,337 bp cDNA sequence of the *ALX4* gene by RT-PCR and the generated sequence data were submitted to the EMBL nucleotide database (GenBank acc. no. AM048630). Similar to human and mouse, a bovine cDNA–genomic DNA alignment confirmed the presence of four *ALX4* exons in cattle. Similar to human *ALX4* we found an ATG start codon at the beginning of the first exon. The stop codon within the bovine *ALX4* transcript was assigned to exon 4 as in the human ortholog. Thus the bovine *ALX4* gene contains an open reading frame of 1,194 bp coding for a protein with 397 amino acids. Alignments between the bovine and human or mouse coding sequence display an identity of about 89.2 and 88.4%, respectively. A multi-spe-

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Bos taurus      MNAETCVSYCESPAAAMDAYYSPVSQSREGSSPFRAYPGGDKFSTTFLSAAAKGQGFGDA  60
Homo sapiens    MNAETCVSYCESPAAAMDAYYSPVSQSREGSSPFRAFPGGDKFGTTFLSAAAKAQGFGDA  60
Mus musculus    MNAETCVSYCESPAAAMDAYYSPVSQSREGSSPFRGFPGGDKFGTTFLSAGAKGQGFGDA  60
                ************************************.:******.******.**.******

Bos taurus      KSRARYGAGQQDPAAPLESGAGARGSFSKFQPQPPAPQPQPP--------------APQP 106
Homo sapiens    KSRARYGAGQQDLATPLESGAGARGSFNKFQPQPSTPQPQPPPQPQPQQQQPQPQPPAQP 120
Mus musculus    KSRARYGAGQQDLAAPLESSSGARGSFNKFQPQPPTPQPPPAP------------PAPPA 108
                ************ *:****.:******.******.:*** *.              .. .

Bos taurus      HLYLQRGACKTPPDGSLKLQEGGGGHNAALQVPCYAKESSLGEPELPPDSDTVGMDSSYL 166
Homo sapiens    HLYLQRGACKTPPDGSLKLQEGSSGHSAALQVPCYAKESSLGEPELPPDSDTVGMDSSYL 180
Mus musculus    HLYLQRGACKTPPDGSLKLQEGSGGHNAALQVPCYAKESNLGEPELPPDSEPVGMDNSYL 168
                **********************..**.************.**********:.****.***

Bos taurus      SVKEAGVKGPQDRASADLPSPMEKADSESNKGKKRRNRTTFTSYQLEELEKVFQKTHYPD 226
Homo sapiens    SVKEAGVKGPQDRASSDLPSPLEKADSESNKGKKRRNRTTFTSYQLEELEKVFQKTHYPD 240
Mus musculus    SVKETGAKGPQDRASAEIPSPLEKTDSESNKGKKRRNRTTFTSYQLEELEKVFQKTHYPD 228
                ****:*.********:::***:**:**********************************

Bos taurus      VYAREQLAMRTDLTEARVQVWFQNRRAKWRKRERFGQMQQVRTHFSTAYELPLLTRAENY 286
Homo sapiens    VYAREQLAMRTDLTEARVQVWFQNRRAKWRKRERFGQMQQVRTHFSTAYELPLLTRAENY 300
Mus musculus    VYAREQLAMRTDLTEARVQVWFQNRRAKWRKRERFGQMQQVRTHFSTAYELPLLTRAENY 288
                ************************************************************

Bos taurus      AQIQNPSWIGNNGAASPVPACVVPCDPVPACMSPHAHPPGSGASGVTDFLSVSGAGSHVG 346
Homo sapiens    AQIQNPSWLGNNGAASPVPACVVPCDPVPACMSPHAHPPGSGASSVTDFLSVSGAGSHVG 360
Mus musculus    AQIQNPSWIGNNGAASPVPACVVPCDPVPACMSPHAHPPGSGASSVSDFLSVSGAGSHVG 348
                ********:************************************.*:*************

Bos taurus      QTHMGSLFGAAGLSPGLNGYELNGEPDRKTSSIAALRMKAKEHSAAISWAT 397
Homo sapiens    QTHMGSLFGAASLSPGLNGYELNGEPDRKTSSIAALRMKAKEHSAAISWAT 411
Mus musculus    QTHMGSLFGAAGISPGLNGYEMNGEPDRKTSSIAALRMKAKEHSAAISWAT 399
                ***********.:********:****************************
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Fig. 2. Alignment of the bovine ALX4 protein with known orthologous ALX4 protein sequences. The sequences were derived from GenBank entries with the accessions NP_068745 (human ALX4), and NP_031468 (mouse Alx4). Identical residues are indicated by asterisks beneath the alignment, while colons and dots represent very similar and similar amino acids, respectively. Conserved protein domains are typed in bold face.

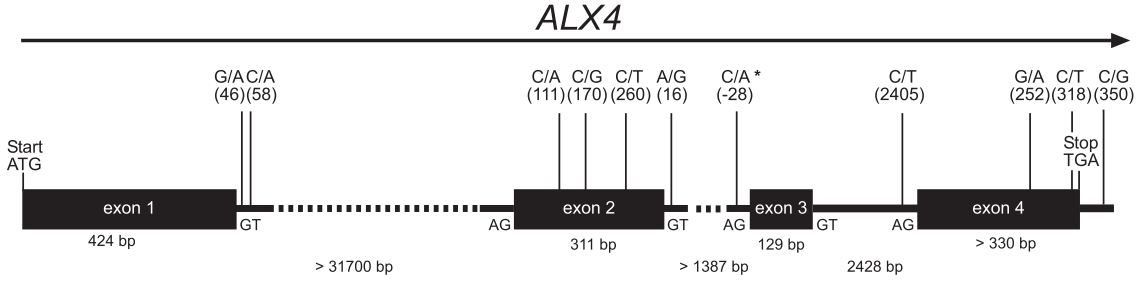

Fig. 3. Genomic structure of the bovine *ALX4* gene. The observed single nucleotide polymorphisms are given above. Numbering of SNPs refers to the position of the polymorphic nucleotide within the given exon or intron, respectively. The SNP in intron 2 marked with an asterisk is situated 28 bp upstream of exon 3.

cies protein alignment revealed that the bovine and murine ALX4 proteins are 14 and 12 amino acids shorter, respectively, than the human protein (Fig. 2). Comparisons between the bovine and human ALX4 protein revealed that this difference is in the region from human residue 103 to 116. The corresponding experimentally derived bovine cDNA sequence length difference in comparison to the human *ALX4* cDNA sequence was confirmed by the independent bovine genomic sequence of the first *ALX4* exon of the bovine genome project. However, protein alignments show a high homology of about 92.5% between the human and bovine proteins. This might probably be due to the fact that the ALX4 protein contains a strongly conserved homeodomain and an OAR or aristaless domain at the N-terminus (Fig. 2).

Using the experimentally derived bovine *ALX4* cDNA sequence, the genomic DNA sequence for all exons could be isolated from the bovine genome project sequence assembly (Fig. 3). A single genomic contig of bovine chromosome 15 contains exon 1 and 2 (GenBank acc. no. NW_928967). This contig includes the sequence of intron 1 spanning 31.7 kb containing four sequence gaps and the 5′-end of intron 2 of 321 bp. The third and fourth exon of *ALX4* could be identified within the subsequent distal contig on chromosome 15 (GenBank acc. no. NW_928968). This contig includes 1,066 bp of the 3′-end of intron 2 and the complete intron 3 of 2,428 bp. Exon sizes range from 129 bp of the smallest exon 3, 311 bp for exon 2, >330 bp for exon 4 to 424 bp of exon 1, respectively (Fig. 3). The canonical splice sites GT-AG of the four bovine *ALX4* exons demonstrate well-defined conserved positions. In the region of the first exon the GC content is 67.1% over 1,566 bp with a CpG_{obs}/CpG_{exp} ratio over 0.70 which corresponds to the generally accepted criteria for a CpG island (Gardiner-Garden and Frommer, 1987). In general the characterization of the transcript and genomic sequences of the bovine *ALX4* gene revealed a con-

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Table 2. Polymorphisms in the bovine *ALX4* gene and genotype distribution

Ref. sequence and position		Position within *ALX4* gene[a]	SNP alleles		Affected animals (n = 14)			Unaffected animals (n = 27)		
			1	2	1/1	1/2	2/2	1/1	1/2	2/2
NW_928967										
32361	A	Intron 1, 46	A	G	2	3	9	9	5	13
32349	C	Intron 1, 58	C	A	14			25	2	
522	C	Exon 2, 111, silent	C	A	9	1	4	20	2	4
463	C	Exon 2, 170, silent	C	G	9		5	21		4
373	C	Exon 2, 260, silent	C	T	14			28	1	
306	A	Intron 2, 16	A	G	14			25		2
NW_928968										
1039	C	Intron 2, –28	C	A	14			26		1
3600	C	Intron 3, 2405	C	T	14			19	8	
3875	G	Exon 4, 252, silent	A	G	14			22	5	
3941	C	Exon 4, 318, silent	C	T	9	4	1	15	11	1
3973	C	Exon 4, 350, 5′ UTR	C	G	14			21	6	

[a] SNP position numbering refers to the position of the polymorphic nucleotide within the given exon or intron, respectively.

served organization with respect to the human and murine orthologs.

Expression analysis of the bovine ALX4 gene

ALX4 expression was detected by reverse transcriptase PCR using primers located in exon 1 and 4 amplifying an 1,188-bp segment of the bovine *ALX4* cDNA. Like in human or mice we only detected expression of the *ALX4* gene in fetal bones. There was no expression in other fetal tissues as lung and liver or in bones, lung and liver tissues from newborn cattle.

Mutation analysis of the bovine ALX4 gene

To evaluate if the coding region of *ALX4* harbors causative mutations for bovine syndactyly we re-sequenced the identified exons with flanking intronic sequences of 14 affected and 27 unaffected individuals. A total of eleven single nucleotide polymorphisms (SNPs) were found in the examined 41 cattle in comparison to the identified cattle genome project reference sequences (Table 2; Fig. 3). Five exonic SNPs located within the coding region represent silent polymorphisms without changing the ALX4 protein sequence (Table 2). Out of the detected sequence variations only five SNPs were detected in the affected cattle in comparison to the bovine reference sequence (Table 2). All affected animals were homozygous A at position 252 in exon 4 instead of G in the reference sequence, but also among the unaffected animals more than 80% of the individuals share this alternative genotype AA (Table 2). Because all mutations detected in affected animals were silent mutations it seems unlikely that the coding sequence variations detected in the *ALX4* gene are causative for bovine syndactyly in the examined breeds. For the four SNPs which were polymorphic within the group of affected animals (Table 2) the individual genotypes are shown in Table 3. Some of the affected cattle show the same genotypes at the SNPs as unaffected animals (Table 2). As affected and unaffected individuals share the same genotypes these polymorphisms do not indicate that the *ALX4* gene is associated with the syndactyly phenotype in the examined cattle. Furthermore syndactyly shows a recessive inheritance so the affected cattle should be homozygous for the mutation under the assumption of a single founder mutation, as suggested in Holstein cattle (Charlier et al., 1996). For the SNP at position 170 of *ALX4* exon 2 all affected Holstein individuals were homozygous but the eight affected calves of a single German Holstein pedigree show two different genotypes (Table 3). The fact

Table 3. *ALX4* SNP genotypes of 14 syndactylous calves

Breed[a]	Animal	Intron 1 46[b]		Exon 2 111[b]		Exon 2 170[b]		Exon 4 318[b]	
GH	8[c]	G	G	C	C	C	C	C	C
GH	9[c]	G	G	A	A	G	G	C	C
GH	10[c]	A	G	C	C	C	C	C	T
GH	11[c]	G	G	A	C	G	G	C	T
GH	12[c]	G	G	C	C	C	C	C	C
GH	20[c]	G	G	C	C	C	C	C	C
GH	21[c]	G	G	A	A	G	G	C	C
GH	29[c]	G	G	A	A	G	G	C	C
GH	511	A	A	C	C	C	C	C	T
GH	91	G	G	C	C	C	C	C	C
GH	101	G	G	A	A	G	G	C	C
CB	31	A	G	C	C	C	C	T	T
CB	331	A	A	C	C	C	C	C	T
FV	611	A	G	C	C	C	C	C	C

[a] GH = German Holstein; CB = Crossbred; FV = German Fleckvieh.
[b] SNP position numbering refers to the position of the polymorphic nucleotide within the given exon or intron, respectively.
[c] Numbering refers to the previously reported pedigree (Drögemüller and Distl, 2006).

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that no further nucleotide differences between the affected and unaffected individuals were observed suggests that *ALX4* is not the gene causing syndactyly. At this time a regulatory mutation within the *ALX4* promoter or within an *ALX4* intron can not be excluded.

In conclusion, *ALX4* seemed to be a plausible candidate gene for bovine syndactyly as the expression is restricted to the developing limb bones. Furthermore, we mapped the gene into the critical region of linked microsatellite markers for bovine syndactyly. The bovine *ALX4* gene was characterized on the genomic and mRNA level and is well conserved with its human and murine orthologs. Several polymorphisms in the bovine *ALX4* gene were identified. But as unaffected and affected 'mulefoot' animals shared identical genotypes, these polymorphisms are obviously not associated with the syndactyly phenotype.

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