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Multiple alleles of *ACAN* associated with chondrodysplastic dwarfism in Miniature horses

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Summary

Chondrodysplastic dwarfism in Miniature horses appeared to be a recessive genetic trait based on the occurrence of affected offspring by normal parents. Dwarf phenotypes vary and range from abnormal abortuses to viable offspring with evidence of skeletal dysplasia. A genome-wide association study implicated a region of ECA1 with dwarfism in Miniature horses. *Aggrecan* (*ACAN*) was a candidate gene in that region, and exons were sequenced to compare DNA sequences for dwarf and non-dwarf horses. Sequencing led to the discovery of variants in exons 2, 6, 7 and 15 associated with dwarfism. The four variants are identified with reference to Ecab 3.0 (GCF_002863925.1) as g.95291270del (rs1095048841), g.95284530C>T (ERP107353), g.95282140C>G (rs1095048823) and g.95257480_95257500del (rs1095048839) and designated here as *D1*, *D2*, *D3**, and *D4* respectively. A previous study at another laboratory reported dwarfism associated with homozygosity for *D3**. Homozygotes for those variants and compound heterozygotes for any combination of those variants always expressed a dwarfism phenotype. However, eight additional horses with dwarfism were found, seven of which were heterozygotes for *D2*, *D3** or *D4*, suggesting the existence of additional *ACAN* alleles causing dwarfism. Among Miniature horses, the combined frequency of *D1*, *D2*, *D3** and *D4* was 0.163, suggesting a carrier rate of 26.2% for alleles causing chondrodysplastic dwarfism.

Keywords equine, exonic variation, recessive disease, teratology

Introduction

Miniature horses are horses (*Equus caballus*) selected for diminutive size. The foundation stock includes many pony breeds. Miniature horses cannot exceed 34 inches at the withers for registration in the American Miniature Horse Association (AMHA 2017) or 38 inches at the withers for registration in the American Miniature Horse Registry (2017), whereas full-sized horses are typically greater than 58 inches at the withers. Recent studies demonstrated that stature among horses is usually the product of many genes (Makvandi-Nejad *et al.* 2012; Signer-Hasler *et al.* 2012; Metzger *et al.* 2013; Tetens *et al.* 2013). Crosses between ponies and full-sized horses produce offspring with an intermediate withers height compared to the parents (Walton & Hammond 1938). Frischknecht *et al.* (2015) identified a variant in the *HMGA2* gene that is common among Shetland ponies and other pony breeds but absent among full-sized horses. This variant is well known to affect stature in other species and is likely a major determinant for the proportionately reduced stature of ponies.

Unfortunately, diminutive size can be accomplished by genetic variants causing conditions characterized as dwarfism. Dwarfism not only affects stature but can also be associated with developmental defects that adversely affect the health of the horse or lead to disproportionate size reduction. Eberth *et al.* (2009) and Metzger *et al.* (2016) identified a series of variants in the *aggrecan* (*ACAN*) gene causing chondrodysplasia-like dwarfism with a recessive mode of inheritance. Rafati *et al.* (2016) described a deletion in the *short stature homeobox (SHOX)* gene causing a dwarfism condition referred to as skeletal atavism with a recessive mode of inheritance. Leegwater *et al.* (2016) described a splice site mutation in *beta-1, 4 galactosyltransferase 7* (*B4GALT7*), causing disproportionate dwarfism among Fresian horses, that also had a recessive mode of inheritance. In other species, variants in at least 14 genes have been reported to cause conditions characterized as dwarfism (reviewed in Boegheim *et al.* 2017).

The subject of this study was dwarfism in Miniature horses caused by nucleotide variations in *ACAN*. *ACAN*

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encodes the core protein of aggrecan, the major proteoglycan component in the extracellular matrix of cartilaginous tissues. The organization of aggrecan molecules combined with a very high negative charge density enable important structural and biomechanical properties of cartilage extracellular matrix. Mutations in *ACAN* are a common cause of chondrodysplasia-like dwarfism among people (reviewed in Dateki 2017; Hauer *et al.* 2017) and domestic animals including mice (Watanabe *et al.* 1994), cattle (Cavanagh *et al.* 2007) and chickens (Li *et al.* 1993). The structures of the gene and protein are similar, although not identical, in all species. In horses, *ACAN* is predicted to contain 17 exons spanning 37 551 bp on ECA1 (94344393–94381944) in ECAB2.0 (Fig. S1). Here, we extend the first studies reported by Eberth *et al.* (2009) describing dwarfism in Miniature horses as caused by a series of mutations in *ACAN*.

Materials and methods

Horses

A total of 196 Miniature horses formed the basis for the discovery of mutations; of these, 139 were AMHA-registered horses with normal appearance and 57 belonged to the pedigree of Miniature horses but were not registered because they exhibited a dwarf phenotype, which is described below. The Miniature horses in this group were not a random assembly but, rather, selected based on (i) a history of producing dwarfs, (ii) having a pedigree from known dwarf producers or (iii) having no indication for dwarfism in their pedigree (controls). Pedigree information was obtained from the studbook of the AMHA registry. Hair, blood or *postmortem* tissue was collected for DNA isolation. The samples included 139 phenotypically normal Miniature horses, 40 Miniature horses that appeared to have chondrodysplastic dwarfism and 17 aborted fetuses with phenotypic characteristics consistent with chondrodysplastic dwarfism. Horses from breeds not reporting the occurrence of dwarf phenotypes, which included 28 Thoroughbreds, 22 American Standardbreds, four American Saddlebreds, four Tennessee Walking horses, four Arabians, one Hackney pony and one Caspian horse, were also tested. After *ACAN* mutations associated with dwarfism were identified, 361 horses identified as Miniature horses by their owners were tested for the variants of *ACAN* as part of a routine screening process for genetic markers.

Phenotyping

Stature reduction is a classic phenotype for dwarfism, but because many of the affected horses were aborted, still born, died before the age of maturity or were characterized before maturity, we could not phenotype by stature. Chondrodysplastic dwarfism in horses was identified based on severely shortened stature relative to horses of that age plus any of the following characteristics: shortened limbs relative to overall body size, bowed forelegs, shortened neck, disproportionately large cranium, compressed faces with large bulging eye sockets with prominent eyes, low nasal bridge, severe underbite, retruded muzzle, cleft palate and protruding tongue, a large abdominal hernia or embryonic loss with a dwarf phenotype of the fetus. A summary of the evaluation for all affected horses is given in Table S1. Phenotyping was done by one of the authors (JE) based on his licensure and expertise with AMHA as a show judge. Phenotypes were based on viewing the animals or from viewing photographs of the horses. Typical phenotypes observed in this study, including a normal Miniature horse (Fig. 1a), two affected horses (1b and 1d) and an aborted fetus (1c), are shown in Fig. 1. The genotypes for each of these horses are defined later in the article: an affected horse with genotype *D2/D2* (Fig. 1b), an aborted fetus with genotype *D1/D1* (Fig. 1c) and an affected horse with genotype *D1/D4* (Fig. 1d).

DNA isolation

DNA from blood or tissue was extracted using Puregene whole blood DNA extraction kits (Gentra Systems, Inc.) and Puregene tissue DNA extraction kits, according to published protocols. Submitted hair samples were processed using 7–10 hair bulbs, according to the method described by Locke *et al.* (2001). Aliquots of the DNA samples were made for working dilutions at concentrations of approximately 150 ng/μl.

SNP genotyping

DNA samples from 46 horses were selected for single nucleotide polymorphism (SNP) genotyping with the Illumina Equine SNP50 chip. The 46 samples included 20 characterized as dwarfs and 26 from Miniature horses with normal appearance but including several known carriers. The set of horses included 22 sets of half-siblings as well as parent–offspring combinations, described in Table S2. SNP50 genotyping was conducted at the Mayo Clinic (Rochester, MN). These data are available at the NAGRP Community File Sharing Platform at https://www.animalgenome.org/repository/pub/UKL2018.0125/.

Analysis of SNP data

PLINK (Purcell *et al.* 2007) was used for analysis of the SNP genotyping data. The Illumina SNP50 chip assayed for 59 349 SNPs. The overall call rate was greater than 95% for all 46 horses, so all 46 were retained for analyses. After filtering individual SNPs for minor allele frequency (>0.05) and call rate (deleted if lower than 90%), the analyses included 40 368 SNPs. The data for dwarf horses (cases) were compared to phenotypically normal horses (controls).

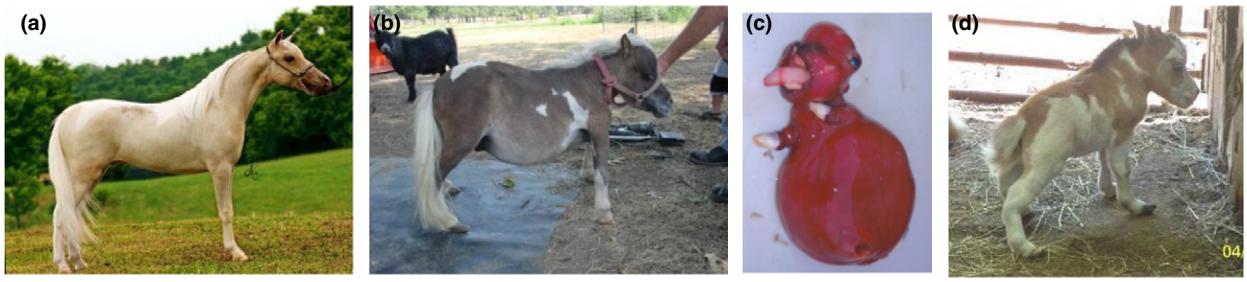

Figure 1 Photographs of phenotypic characteristics for Miniature horses possessing variants of the *ACAN* allele: (a) Phenotypically normal, (b) dwarf Miniature horse with a *D2/D2 ACAN* genotype, (c) aborted fetus with *ACAN* genotype *D1/D1*, (d) Miniature horse with a *D1/D4 ACAN* genotype.

An association study with a Monte-Carlo-based approach (5000 permutations) was run to compare differences in the occurrence of SNPs among the case and control horses. The permutation option (EMP2) corrected for the large number of comparisons being made. *P*-values less than 0.05 for an EMP2 were considered significant and the region selected for further analyses. The SNPs from the region with statistical significance were further tested using the haphase option to identify haplotypes associated with the trait and to deduce the mode of inheritance.

Predicted structure of *ACAN* in horse

The annotation for *ACAN* from ENSEMBL release 87 of ECAB 2.0 was used as the reference for exon and intron differentiation. However, a new reference genome was produced for the horse just prior to submission of this manuscript, and genome coordinates were converted from Ecab 2.0 (GCF_000002305.2) to Ecab 3.0 (GCF_002863925.1) using the REMAP tool from NCBI (https://www.ncbi.nlm.nih.gov/genome/tools/remap/docs/api). *ACAN* is predicted to contain 17 exons spanning 38 055 bp on ECA1 (95 255 771–95 293 825). The protein sequence reference is ENSECAP00000005919.

DNA sequencing

As described in the Results section, *ACAN* was identified as a candidate gene based on a GWAS and selected for exon sequencing. Primer sets were designed for 17 exons defined by ENSEMBL annotation (release 87). Primers and their locations are listed in Table S3. All exons were sequenced except for the large variable repeat region, including repeats 63 bases in length, in exon 12, which spans over 2000 bases. Exon coordinates are reported using the ECAB 3.0 assembly (GCF_002863925.1). Each amplicon was sequenced using Sanger sequencing based on the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems), according to manufacturer's instructions. The resulting sequence product was cleaned using Centri-Sep columns (Princeton Separations, Inc.) and read on an ABI 310 genetic analyzer (Applied Biosystems). The results were analyzed using the VECTOR NTI ADVANCE 10.3 software package using the CONTIGEXPRESS ALIGNMENT program (Invitrogen Corp.).

Variant detection

Following the discovery of variants, SNPs were detected using Sanger sequencing or Taqman® assays. Deletions were detected using Taqman® assay or agarose gel electrophoresis. To detect the presence and distribution of variants among other normal and dwarf horses, including horses of other breeds, custom Taqman® SNP Genotyping Assays (Applied Biosystems) were designed for the three variants—the exon 2 deletion g.95291270(A/−), the exon 6 SNP g.95284530(C/T) and the exon 7 SNP g.95282140 (C/G)—using FILEBUILDER 3.1 software (Applied Biosystems). These assays were run on a 7500 HT Fast Real Time-PCR System (Applied Biosystems). The fourth variant, in exon 15 with a 21-bp deletion, was amplified by PCR, and the amplicon product was quantified on a 2% agarose gel to determine the existence of sequence size variants. The primer sequences used for these assays are described in Table S4. Any ambiguous results from the Taqman® assays or gel electrophoresis were subsequently analyzed by Sanger sequencing, as previously described.

Assessing the impact of genetic variants

The predicted effects for variants were calculated using SIFT (Kumar *et al.* 2009) and POLYPHEN-2 (http://genetics.bwh.harvard. edu/pph2/) (Adzhubei *et al.* 2010) for SNPs and PROVEAN for SNPs and indels (Choi *et al.* 2012). These programs assess the likelihood that changes in amino acids will alter protein function based on the physical and chemical properties of the amino acids.

Alignment of amino acid sequences in *ACAN* exon 6 across seven species

Predicted amino acid sequences were obtained for a region of *ACAN* exon 6 for *Equus caballus* (F7C3C6), *Homo sapiens* (P16112), *Canis familiaris* (Q28343), *Bos taurus* (P13608),

Mus muscalus (Q61282), *Gallus gallus* (P07898) and *Sus scrofa* (Q29001).

Results

Genome-wide association study

Comparison of SNP distribution for the 20 chondrodysplastic dwarfs to that of the 26 normal horses identified statistically significant differences in the distribution of SNPs on ECA1. The design of this part of the project was not intended as a classical case–control study with unrelated animals; however, the results implicated a candidate gene as described below. The most significant SNP was *BIEC2-38994* on ECA1 at position 95 126 445 with $P = 1.22 \times 10^{-6}$ and EMP2 value of 0.003. Haplotype analyses were conducted, comparing cases and controls using the hap-phase option within PLINK. The strongest association occurred for a haplotype region of 76 413 bp based on five SNPs: *BIEC2-38884*, *BIEC2-3895*, *BIEC2-38970*, *BIEC2-38986* and *BIEC2-38994*, spanning ECA1 from 95 050 030 to 95 126 445. Although we expected to find homozygosity for a single haplotype among dwarfs, given that this form of dwarfism was initially thought to be a simple recessive trait with a single founder, four haplotypes were observed among the dwarf horses. Of the 20 dwarfs, six were homozygous for one haplotype whereas the remainder were heterozygous for different combinations of all four haplotypes, as described in Table S5. Among the normal horses, five haplotypes were observed including the four found among the dwarf horses, although at divergent frequencies, when comparing the two groups.

The haplotype region associated with dwarfism was 129 116 bp from a gene homologue known to cause dwarfism among humans, mice, chicken and cattle, specifically *aggrecan* (*ACAN*) (Li *et al.* 1993; Vertel *et al.* 1993; Watanabe *et al.* 1997; Gleghorn *et al.* 2005; Cavanagh *et al.* 2007; Tompson *et al.* 2009; Stattin *et al.* 2010). Based on the proximity to *ACAN* and its identity as a candidate gene for dwarfism, exons for *ACAN* were sequenced and compared between affected and unaffected individuals.

Genotype variants

Dwarf horses found homozygous for the aforementioned GWAS haplotype had their *ACAN* exons sequenced, leading to the discovery of homozygosity for a non-synonymous SNP in exon 6 (g.95284530C>T) (Table 1). This SNP causes the amino acid change p.Val424Met. The haplotype containing this SNP was designated *ACAN-D2*. Analyses by POLYPHEN-2, SIFT and PROVEAN suggested either 'no effect' or a 'possibly damaging' effect from the p.Val424Met polymorphism. Because the prediction programs did not assign a serious risk to this amino acid change, this peptide region of *ACAN* was aligned across six other species and showed moderate conservation of the amino acid with the valine residue present in four of the six other species (Table S6).

Next, exons from dwarf horses heterozygous for the *ACAN-D2* allele were sequenced, leading to the discovery of three additional variants potentially causing dwarfism, also described in Table 1. *ACAN-D1* was characterized as a single nucleotide deletion in exon 2 (g.95291270del) resulting in a stop codon (Table 1). Analysis with PROVEAN predicted the mutation to be deleterious (Table 1). *ACAN-D3** was found to harbor a non-synonymous SNP substitution in exon 7 (g.95282140C>G) (Table 1). POLYPHEN-2, SIFT and PROVEAN predicted this variant to be 'probably damaging' or 'deleterious' (Table 1). *ACAN-D4* was characterized by a 21-base deletion in exon 15 (g.95257458_95257500del) (11), which PROVEAN analysis indicated was probably 'deleterious' (Table 1). *ACAN* haplotypes without any of these specific four variants were designated as *ACAN-N (N* for *non-dwarf haplotype)*. Additional SNPs were discovered during the sequencing investigations; however, their occurrences were not correlated with dwarfism (Eberth 2013). Hereafter, the five identified alleles are referred to as *N*, *D1*, *D2*, *D3** and *D4* based on SNPs at these four sites with *N* representing the absence of SNPs characterizing *D1*, *D2*, *D3** and *D4*.

Table 1 *ACAN* variants associated with dwarfism in horses and predictions from SIFT, POLYPHEN-2 and PROVEAN for effect on the protein.

Allele: *ACAN*–	Pheno-type	Genomic variant (ECA1, Ecab 3.0, GCF_002863925.1	Exon	Protein NSECAP00000005919	dbSNP#	Predictions POLYPHEN-2	SIFT	PROVEAN
N	Normal	None						
D1	Dwarf	g.95291270del	2	p.Lys82fx	rs1095048841	NA	NA	−13.081 deleterious
D2	Dwarf	g.95284530C>T	6	p.Val424Met	ERP107353	0.927 possibly damaging	0.07 moderate	−0.874 neutral
*D3**	Dwarf	g.95282140C>G	7	p.Ala505Pro	rs1095048823	0.994 probably damaging	0.00 deleterious	−4.833 deleterious
D4	Dwarf	g.95257458_95257500del	15	p.Phe2017–ASP2023del	rs1095048839	NA	NA	−44.787 deleterious

Distribution of *ACAN* alleles among horses

The distributions of *ACAN* alleles were investigated among the population of 196 Miniature horses, some of which exhibited dwarfism (Table 2). All compound homozygotes and compound heterozygotes for *D1*, *D2*, *D3** and *D4* listed in Table 2 exhibited characteristics of chondrodysplastic dwarfism or were aborted fetuses. Among the 17 aborted fetuses, 15 had the *D1* allele. Only one viable compound heterozygote (*D1*/*D4*) with the *D1* allele was identified.

A panel of horses from other breeds was tested for the existence of any of the putative causative *ACAN* mutations. Horses from other breeds that have not reported the occurrence of dwarf phenotypes included 28 Thoroughbred, 22 American Standardbred, four American Saddlebred, four Tennessee Walking horses, four Arabian, one Hackney pony and one Caspian horse. None of the 64 horses from these seven breeds possessed alleles *D1*, *D2*, *D3** or *D4*.

Evidence for additional *ACAN* variant causing dwarfism

During the study of dwarf horses, eight individuals were identified as chondrodysplastic dwarfs that possessed only one ($n = 7$) or none ($n = 1$) of the *D1*, *D2*, *D3** and *D4* alleles. This suggests the existence of yet additional mutation(s) of *ACAN* causing dwarfism.

Prevalence of *ACAN* mutations among Miniature horses provided by horse owners

The frequencies of the five known *ACAN* alleles among 361 horses submitted for testing by owners of Miniature horses are shown in Table 3. Only one of the horses submitted for testing was phenotypically identified as a dwarf; the rest had normal phenotypes. Among the normal horses, none were found with more than one of the *D1–D4* alleles. The cumulative frequency of the dwarfism-causing alleles was 0.163 (q), suggesting a carrier rate ($2pq$) of 26.2% and a predicted rate of affected horses (q^2) as 2.7%.

Table 2 *ACAN* genotypes for 197 American Miniature Horse Association horses selected from herds known to be affected, including 139 normal individuals, 41 chondrodysplastic dwarfs and 17 aborted fetuses.

	Phenotypes		
Genotypes	Term live dwarf	Aborted dwarf fetus	Normal
N/N	1	0	62
N/D1	0	0	27
N/D2	4	0	44
*N/D3**	1	0	4
N/D4	2	0	2
D1/D1	0	3	0
D1/D2	0	9	0
*D1/D3**	0	2	0
D1/D4	1	1	0
D2/D2	18	1	0
*D2/D3**	7	1	0
D2/D4	5	0	0
D3/D3**	0	0	0
D3/D4*	1	0	0
D4/D4	0	0	0
Totals	40	17	139

Table 3 Gene frequencies of the *ACAN* alleles among 361 randomly selected horses submitted for testing by private horse owners.

Allele	Frequency
N	0.84
D1	0.03
D2	0.09
*D3**	0.02
D4	0.03

Discussion

Anecdotal reports and the family studies reported here suggested familial dwarfism of Miniature horses with a recessive mode of inheritance. Miniature horses characterized as chondrodysplastic dwarfs exhibited a wide range of phenotypic variation, so initially we suspected multiple loci may be involved. Based on a genome-wide association study using the Illumina SNP50 chip, *ACAN* was identified as a potential candidate gene for the condition in some of the Miniature horses. As we began sequencing *ACAN* for affected horses, it became apparent that the simplest explanation was multiple functionally important loci within *ACAN*. In every case for which a horse was found to be homozygous for one of these four variants and in every case for which a horse was heterozygous for any combination of the *D1*, *D2*, *D3** and *D4* alleles, the individual exhibited chondrodysplasia-like dwarfism or was aborted during pregnancy (Table 1). At the same time, eight chondrodysplastic dwarfs were found that were not compound heterozygotes for *D1–D4*. Seven of those were carriers of *D2*, *D3** or *D4*, suggesting that additional variants of *ACAN*, and possibly even other loci, may contribute to the condition. Indeed, work is currently underway to characterize a fifth variant of *ACAN* (putative *D5*) that contributes to dwarfism (unpublished data).

ACAN-D1 is a deletion in exon 2 (Table 1). *ACAN* exon 2 contributes to the Globular 1 domain of *ACAN*. The result of the deletion was the predicted creation of a stop codon and failure to produce a functional protein.

ACAN-D2 is a non-synonymous mutation in exon 6 resulting in a missense mutation predicted to replace the amino acid valine with methionine. The predictions for the effect of the amino acid changes were comparatively mild, ranging from 'neutral' to 'possibly damaging' (Table 1). We compared the amino acid sequences in this region for several species (Table S6) and observed modest conservation of the region. On one hand, the predictions of a modest

effect are consistent with the observation that horses homozygous for the *D2* allele are usually viable, and this is the most common of the four dwarfism alleles; the effect of this mutation is less deleterious than those for *D1*, *D3** and *D4*. On the other hand, we did not fully sequence exon 12, introns or flanking regions of the gene, and the mutation responsible for this trait could lie in that region. The variant described here may simply be a marker in linkage disequilibrium with the causative allele. If this is the case, then we should see some horses with the *ERP107353* variant in association with other alleles but not exhibiting dwarfism.

*ACAN-D3** is a single-base missense mutation in exon 7 producing a change of the amino acid alanine to proline. Metzger *et al.* (2016) previously reported homozygosity of this variant as the cause of dwarfism in a Shetland pony. This missense mutation may alter the structural and possibly functional integrity of the interglobular domain encoded by exon 7 and involved in the physiological turnover of aggrecan. Previously, a deletion in exon 11 was ascribed as the cause of dwarfism in a set of horses and the haplotype identified as *ACAN-D3* (Eberth 2013). Subsequently this result was found to be a technical artifact; the actual cause of dwarfism in these horses was found to be the variant in exon 7, as described above and reported by Metzger *et al.* (2016). Therefore, this allele has been assigned the designation *D3**, signifying it is a different allele from that reported in the thesis (Eberth 2013) but responsible for the phenotype originally ascribed to D3.

ACAN-D4 is associated with a 21-base deletion in exon 15. This variant is predicted to be deleterious (Table 1).

The different phenotypic consequences for different genotypes are shown in Table 2. Of the 17 aborted fetuses tested, 15 had at least one *D1* allele. Furthermore, only one viable dwarf horse, shown in Fig. 1d, had a *D1* allele (*D1*/*D4*). The extreme consequences for the presence of the *D1* allele are consistent with the prediction that this variant would lead to complete failure to produce an aggrecan protein. Conversely, most of the viable dwarfs had the *D2*/*D2* genotype. This is also consistent with the more modest effect one might expect for a missense mutation leading to a functional, yet imperfect, aggrecan protein.

The photo of an aborted fetus that was subsequently genotyped as *D1*/*D1* is shown in Fig. 1c. The appearance is strikingly similar to the 'bulldog calves' in Dexter cattle with a mutation in *ACAN* (Cavanagh *et al.* 2007). Aborted fetuses were also genotyped as *D2*/*D2* except for one with genotype *D2*/*D3**. No horses were observed in this study with genotypes *D3**/*D3** or *D4*/*D4*, and this may be due to the low frequency of the *D3** and *D4* alleles. Metzger *et al.* (2016) did observe a viable individual homozygous for *D3**.

The combined gene frequency of the four dwarfism alleles was found to be 0.163, which would predict a carrier rate of 26.2%. This seems high for sequence variants within a gene that have a deleterious effect on the phenotype and suggests there may be some offsetting positive selection for heterozygotes. Therefore, it remains to be seen whether or not these variants are true recessive alleles or if they exert some phenotypic effect on carriers, such as the possibility of contributing to small stature, that makes them more attractive to breeders.

Finally, variants of *ACAN* are well known as a cause of dwarfism in other species. A survey of human cases of idiopathic short stature identified variants in *ACAN* as the cause in six out of 428 families. In a review of the literature on stature and *ACAN* variants for people, 25 non-synonymous mutations were identified, including those in exons 2, 3, 4, 6, 7, 8 9, 10, 12, 14, 15 and 16 (Dateki 2017); of those 25, 24 exhibited a dominant or partial dominant mode of inheritance, the only exception being one of two variants reported for exon 15. For cattle, two *ACAN* variants were identified as a cause of dwarfism (Cavanagh *et al.* 2007). Homozygotes and compound heterozygotes for the two variants resulted in 'bulldog calves' aborted mid-gestation. Carriers of either variant were mildly affected, exhibiting disproportionate dwarfism. In mice, homozygotes for a 7-bp deletion within exon 7 were severely affected and died shortly after birth, whereas heterozygotes appeared normal at birth but developed symptoms of dwarfism with age (Watanabe *et al.* 1994, 1997). In chickens, a nonsense mutation in exon 12, called nanomelia, produces a recessively inherited disorder causing disproportionately small limbs (Li *et al.* 1993; Vertel *et al.* 1993; Primorac *et al.* 1994). Exons 2, 6, 7 and 15 were implicated in this study of the horse, and variants found in those exons resulted in a recessive form of dwarfism with homozygotes and compound heterozygotes exhibiting chondrodysplastic dwarfism. In light of observations for reduced stature in carriers in other species and in light of the high frequency of carriers among Miniature horses, these results suggest that carriers may have a phenotype selected by breeders, possibly reduced stature but also possibly some other physiognomic trait. The ability to identify carriers for these *ACAN* variants makes it possible to investigate this hypothesis.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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Supporting information

Additional supporting information may be found online in the supporting information section at the end of the article.

Figure S1 Schematic diagram of the intron/exon structure of *ACAN* and its relationship to the protein feature of aggrecan adapted from ENSEMBL annotation release 87 of ECAB 2.0. Exons possessing variants associated with dwarfism are colored blue.

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Table S1 Characteristics of horses identified as dwarfs; accession number, *ACAN* genotype and 12 diagnostic, phenotypic characteristics.
Table S2 Relationship of 46 samples submitted for the genome-wide association study.
Table S3 Primers for exons 1–17 of *ACAN* (5′–3′), coordinates from Ecab 3.0 (GCF_002863925.1).
Table S4 Primer sequences for *D1*, *D2*, *D3** and *D4 ACAN* variants.
Table S5 Dwarf haplotypes (*D1–D4*) in relation to genome-wide association study haplotypes (not reported) for ECA1.
Table S6 Comparison of amino acid sequences in the region defined by the *D2 ACAN* allele. Refseq numbers for protein sequence identify the source of the proteins (*Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Bos taurus*, *Sus scrofa*, *Canis lupus* and *Equus caballus*) *N* allele and the *D2* allele.