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Genetic factors of horn-related traits in small ruminants with special reference to the polled intersex syndrome (PIS) in the goat

INAUGURAL-DISSERTATION

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Wenn wir wüssten, was wir tun, würde das nicht Forschung heißen, oder?

~Albert Einstein~

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ADM	Anaesthesia delegation model
AMH	Anti-Mullerian Hormone gene
ARHGAB15	Rho GTPase activating protein 15 gene
bp	basepair(s)
cm	centimeter
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DHH	Desert hedgehog gene
DMRT1	Doublesex and mab-3 related transcription factor 1 gene
DSD	Disorder(s) in sexual development
EMBL	European Molecular Biology Laboratory
e.g.	exempli gratia
ERG	ETS transcription factor ERG gene
FOXL2	Forkhead box L2 gene
GATA4	GATA binding protein 4 gene
GTDC1	Glycosyltransferase like domain containing 1 gene
HOXD1	Homeobox D1 gene
HSC	hematopoietic stem cells
ISAG	International Society for Animal Genetics
Kb	kilobase(s)
KCNJ15	potassium inwardly rectifying channel subfamily J member 15 gene
Mb	Megabase(s)
MOWS or MWS	Mowat-Wilson Syndrome
NRXN1	Neurexin 1 gene
PCR	Polymerase chain reaction
PIS	Polled Intersex(uality) Syndrome
PISRT1	PISRT1 lncRNA
PMS	Polled and Multisystemic Syndrome
RXFP2	Relaxin family peptide receptor 2 gene
SFRP4	Secreted frizzled related protein 4 gene
SIM1	SIM bHLH transcription factor 1 gene
SNP	Single nucleotide polymorphism
STIM1	Stromal interaction molecule 1 gene
SUED	split upper eyelid defect
TALEN	Transcription activator-like effector nuclease
TWIST1	Twist family bHLH transcription factor 1 gene
U.S.	United States
UTR	Untranslated region
WNT3	WNT family member 3 gene
WT1	WT1 transcription factor gene
ZEB2	Zinc finger E-box binding homeobox 2 gene

1. SUMMARY

Bovidae belong to the family of ruminants which unite a range of even-toed ungulates like cattle, sheep, goat and antelopes. The group of pecorans is very heterogeneous in their phenotypic appearance, but have a common feature, the horns, which appear in pairs. The characteristics associated with the presence or absence of horns are as diverse as the group itself. In livestock, especially cattle and goats, polledness is often seen as a desirable trait, as it can reduce the risk of injury to humans and flockmates. In order to achieve this, young horned animals were or still are dehorned. In view of the ever-increasing controversy and tightening of the legal situation in this regard, for example in Germany, breeding and selection for genetically polled livestock offers a possible alternative.

While knowledge about inheritance and underlying gene variants for horn-specific traits is extensive for cattle, there are still some gaps in knowledge for small ruminants (sheep and goats) - even for long-known phenomena. The polled intersex syndrome (PIS) in goats, which describes the fact that female, homozygous polled goats are infertile hermaphrodites, has been described since the 1940s. Due to the wide variability in phenotypic expression, affected animals are often difficult to identify. This restricts breeding for polledness in goats. Despite the published association with an 11.7-kb deletion on chromosome 1 that affects the transcription of the *PISTR1* and the *FOXL2* gene, it was not possible for a long time to develop a genetic test for the early detection of affected individuals that could be used in practice. The identification of a complex rearranging structural variant, consisting of the named deletion in combination with an inverse inserted duplication, associated with PIS was made possible through the use of long-read whole genome sequencing in the context of this work. This finding made it possible for the first time to develop an early applicable genetic test to identify all three possible genotypes, as well as the sex. Subsequent publications have confirmed this variant for all goat breeds examined worldwide. Also for the trait polledness in sheep a causal variant in form of an 1.78-kb sized insertion in the 3‘-UTR region of the *RXPF2* gene on chromosome 10 has been published for some time. However, it has been shown several times that this does not segregate with the trait in all breeds. This applies in particular to breeds with variable or sex-specific horn status. This was mainly confirmed for the Icelandic sheep in this study. In sheep, the *RXFP2* gene has been shown to be not only associated with the presence/absence of horns but also with other traits related to horns, such as size and shape. Horn size showed some association with a previously published *RXFP2* variant in the Icelandic sheep as well. The inclusion of polycerate Icelandic sheep in the present work confirmed the segregation of a previously published 4-bp insertion in *HOXD1* (ovine chromosome 2) with this trait, but also brought new findings. For the first time, this insertion was detected in polled individuals of polycerate origin. And a simultaneous observation of the *HOXD1* and *RXFP2* variants mentioned allows the assumption that polledness in Icelandic sheep of polycerate origin is not controlled by the *RXFP2* insertion described.

This work has helped to solve some unknowns, especially with regard to the PIS of the goat. However, insights were also gained that raise further questions, such as polledness in sheep of polycerate origin. These should be investigated more intensively in other breeds and larger samples in the future. The further development of molecular genetic methods can also help to clarify further horn-specific traits and verify initial indications, like those found in this study.

2. INTRODUCTION

Domestication of the livestock species cattle, sheep and goat in the Middle East (Fertile Crescent) laid the foundation for today's agriculture, but also for the emergence of the current diversity of breeds and traits of farm animals (Diamond, 2002).

Through intensified husbandry of the mentioned species, humans have come into closer contact with them. The use of livestock was beneficial for mankind (Ahmad et al., 2020). But the close contact with the animals also revealed the risks for humans and flockmates. To minimize the increased risk of injury, but also payout losses for reduced meat quality due to bruising (Youngers et al., 2017; Mendonça et al., 2016) lower milk yield and quality due to udder injuries or bruising on valuable cuts, dehorning of young animals is used especially in cattle farming.

Since this procedure is associated with stress and potentially also pain for the animals, it is partly regulated by law, and in the case of goats in Germany, for example, it is already prohibited completely (according to the German Animal Welfare Act §6 paragraph 1). Breeding for polledness can be seen as an animal friendly alternative, although this possibility is also associated with restrictions, especially in goats. In this species, polledness is associated with interferences in sexual development, known as polled intersex syndrome (PIS) (Asdell, 1944; Eaton, 1945). Prior to the start of this work, there is no genetic test available to help identify affected animals as early as possible. While in cattle the knowledge about the genetic factors underlying polledness is great, in small ruminants many pieces are still missing. In sheep, for example, an 1.78 kb-sized insertion on chromosome 10 associated with polledness has been known for several years, but this does not segregate with the trait in all breeds (Wiedemar and Drögemüller, 2015; Lühken et al., 2016). Other variants are not yet known. Consequently, information on the functional basis of such horn-related traits of interest for breeding and knowledge on possible targets for selective breeding is still required.

2.1. Development, morphology and inheritance of horns (and scurs)

Horns are referred to as cranial appendages or headgear in bovine species, which have to be delimited from the antlers in cervids, ossicones in giraffids and pronghorns in antelopes – all belonging to the family of ruminants (Davis et al., 2011). Little is known about the evolution of this manifold trait (Davis et al., 2011). The occurrence of polledness in cattle can be traced back to ancient times. Depictions of polled cattle have been found on petroglyphs and in burial sites in ancient Egypt. It is estimated that those polled individuals had a very high value, as they were never depicted as working animals (Schafberg and Swalve, 2015). Depictions of hornless goats can also be found on ancient (approx. 3000 BC) Egyptian illustrations (reviewed in Amills et al., 2017).

The horn morphology varies among species, which becomes evident when examining the non-domesticated relatives of small ruminants (Figure 1), as well as between breeds. The latter is particularly evident in sheep, but also known in cattle (Figure 2). Individuals can express a variety of horn forms (Table 1), ranging from small and slightly curved ones up to massive spiral ones. The diameters can be rather rounded or angular. Sometimes forms also differ between the sexes of one breed. Additionally there is a special semi-form known in sheep and cattle. The small, mis-shaped and flexible scurs, which are, in contrast to normal horns, not attached to the skull (Gehrke et al., 2020b; White, W.T. and Ibsen, H.L., 1936; Long and Gregory, 1978). Their existence in goats is not proven, but breeders sometimes report hornlike structures for this species as well (personnel communication, Figure 3). Nevertheless, a common feature of all horns in different species is their origin in the cells of the neural crest (Guo et al., 2021).

Figure 1: Various horn shapes and sizes in non-domesticated representatives of small ruminants.
All pictures were taken in the Museum of Natural History in Vienna, Austria.
1. line (from left to right): Argali (dt.: Argali; Ovis ammon), Dall's sheep (dt.: Dallschaf ; Ovis dalli), Mouflon (dt.: Mufflon; Ovis gmelini musimon), Siberian bighorn sheep (dt.: Schneeschaf; Ovis nivicola), Bighorn sheep (dt.: Dickhornschaf; Ovis Canadensis).
2. line (from left to right): Alpine ibex (dt.: Alpensteinbock; Capra ibex), Siberian ibex (dt.: Sibirischer Steinbock; Capra sibirica), Pyrenean ibex (dt.: Pyrenäensteinbock; Capra pyrenaica), Kuban tur (dt.: Westkaukasischer Steinbock; Capra caucasica), Daghestan tur (dt.: Ostkaukasischer Steinbock; Capra cyclindricornis), Agrimi (dt.: Kretaziege; Capra aegagrus cretica).
3. line (from left to right): Japanese serow (dt.: Japanischer Serau; Capricornis crispus), Sumatran serow (dt.: Südlicher Serau ; Capricornis sumatrensis)
4. line (from left to right): Bharal (dt.: Blauschaf; Pseudois nayaur), Mountain gaot (dt.: Schneeziege; Oreamus americanus), Himalayan tahr (dt.: Himalaya-Tahr; Hemitragus jemlahicus).
5. line: Barbary sheep (dt.:Mähnenspringer ; Ammotragus lervia).

Horns are skin organs enclosed in a keratin sheath, which develop from the epithelial bud and the proliferation of the connective tissue underneath. The bony horn cone formed in this way is connected to the frontal sinus in the first year of life at the latest; in small ruminants this happens at a younger age than in cattle. The blood supply to the horn is provided by the terminal branches of the temporal artery and vein (A. and V. temporalis). Innervation is mainly through the zygomaticotemporal nerve (N. zygomaticotemporalis) (König and Liebich, 2019).

Table 1: Different horn forms and example breeds from different species displaying them. Please note, the list does not claim to be complete and the scheme of the sheep head is used for every form for simplification, information about horn forms was taken from Porter et al., 2016.

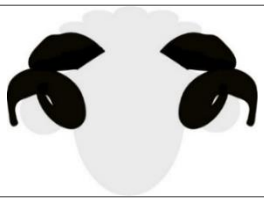
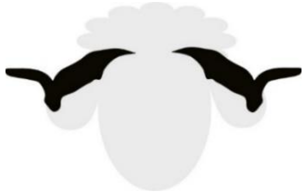
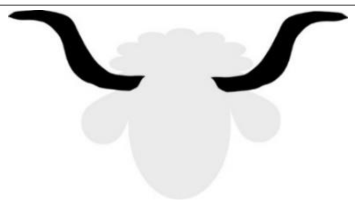
Horn form	Example breed (species)	Scheme
Spiral	Skudde (*Ovis aries*), Grey Horned Heath (*Ovis aries*)	
Horizontal screwed	Roux du Valais (*Ovis aries*), Valais Blacknose Sheep (*Ovis aries*)	
V-formed and twisted	Racka sheep Zackelschafe (*Ovis aries*)	
Crescent curved (a: upwards) (b: backwards)	Peacock goat (*Capra hircus*), Saanen goat (*Capra hircus*) Cameroon sheep (*Ovis aries*)	 a  b
Curved outwards	Capra Sempione (*Capra hircus*)	
Lyre-shaped	Scottish Highland Cattle (*Bos taurus*) Hungarian Grey (*Bos Taurus*)	
Short and strong, bent upwards	Hérens Cattle (*Bos Taurus*)	

Figure 2: Variety of horn shapes in the cattle breed Angler Rotvieh.
Please note the most common shape being displayed in the upper row, while also shapes like bend inwards horns (second row, left) or horns extending far outward with horn tips pointing upwards (second row, middle and right) can be found l. All cows come from the same flock.

Figure 3: German Improved White buck with horn crusts.
According to the breeder, the animal had shed its approximately 5 cm horn stubs, considered as scur-like structures, a few days bevor the picture was taken. However, these would grow back, according to the statement. (Picture: G. Lühken)

2.1.1. Inheritance

Clarification of the challenging question of the inheritance of polledness in ruminants has occupied many scientists since the 1900s. Nevertheless, it is now considered proven, that horns represent the wild type in cattle and that this trait is recessive to polled (P) (Aldersey et al., 2020). The situation is similar for goats in which polledness (P) dominates horns (p) (Asdell und Crew 1925), but different for sheep, where differences between breeds might be obvious. For instance for some breeds, polledness is described as recessive trait (Clutton-Brock and Pemberton, 2004; Pickering et al., 2009). Whereas Johnston at al. (2011) observed a sex-dependent type of gene action in which the horned allele is dominant in male Soay sheep and the two alleles being additive in female Soay sheep. For the merino population polledness is described as dominant (Dolling, 1960).

Due to the heterogeneity of the molecular causes of inherited polledness in the different species it is suggested that the underlying mutations occurred independently.

While in cattle (*Bos Taurus* OMIA:00483-9913 and *Bos indicus* OMIA:00483-9915 (Nicholas and Tammen, 1995)) knowledge about the traits underlying gene variants is great (Medugorac et al., 2012; Medugorac et al., 2017; Gehrke et al., 2020b; Gehrke et al., 2020a; Lamb et al., 2020; Aldersey et al., 2023), it is still incomplete in sheep (OMIA: 000483-9940 (Nicholas and Tammen, 1995)). To date, all studies in various sheep breeds with different origins, using multiple, ever-evolving methods, point to a central role of the *relaxin family peptide receptor 2* (*RXFP2)* gene in the inheritance of horn-related traits in this species (e.g. Dominik et al., 2012; Johnston et al., 2011). However, only the 1.78 kb-sized insertion into the 3'-UTR region or *RXFP2* has been confirmed causal for polledness in breeds with sex-independent horn status (Wiedemar and Drögemüller, 2015; Lühken et al., 2016). Additional variants in or close to *RXFP2* gene can only be referred to as markers, often just for specific breeds, for horn size and shape (Pan et al., 2018; Johnston et al., 2010) or the absence of horns (Duijvesteijn et al., 2018). Therefore, not just the existence of horns in sheep (*Ovis aries*) is associated with *RXFP2*, but also morphological horn attributes. For example a haplotype consisting of two SNPs was found to be associated with higher length and a spiral form in various Chinese breeds (Pan et al., 2018). However, not in Thinhorn sheep (*Ovis dalli*, Figure 1, first line) in which two loci one on ovine chromosome 2 and 3 each, where found to be associated with horn length (Sim and Coltman, 2019).

The locus for polledness in goats was mapped to the distal end of chromosome one (Vaiman et al., 1997). The variant underlying polledness in goats is connected with intersexuality (for details refer to chapter 2.5.1.1.) and is described as an 11.7 kb-sized deletion affecting the transcription of two genes - *polled intersex syndrome regulated transcript 1* (*PISTR1*) and *forkhead box L2* (*FOXL2*) (Pailhoux et al., 2001).

2.2. Special traits related with horn status

In addition to the occurrence of a solid pair of horns, additional types have been described. They refer to the development of horn-like structures or the occurrence of a large number of horn pairs.

2.2.1. Scurs

In contrast to horns, scurs are defined as small bony structures that are not firmly fused with the skull. Sometimes they are also referred to as knobs. The scurs phenotype is variable in size (Dove, 1935). Its occurrence has been proven in both sheep (Ibsen, 1944) and cattle (Long and Gregory, 1978; Capitan et al., 2009); there is no confirmation for goats, but breeders report the occurrence of small, unattached horns in this species as well (Figure 3). Although not fully elucidated yet, most of the evidence on this sex-influenced trait is available in cattle (OMIA: 000894-9913 (Nicholas and Tammen, 1995)). The long accepted mode of inheritance of scurs being a dominant trait in bulls, while two copies of allele *Sc* are required in females to express the trait (White, W.T. and Ibsen, H.L., 1936) has meanwhile been disproven (Gehrke et al., 2020b). However, it was confirmed that animals carrying scurs are heterozygous for one of the known polled variants (Gehrke et al., 2020b). The scurs locus was mapped to the bovine chromosome 19 (Asai et al., 2004) but recently a genome-wide linkage mapping showed significant loci on two chromosomes, giving rise to the hypothesis of scurs being a polygenic trait (Gehrke et al., 2020b; Mariasegaram et al., 2010).

A phenotypically similar but still different trait, named type II scurs (OMIA: 001593-9913 (Nicholas and Tammen, 1995)), was exclusively found in a Charolais family. In contrast to type I scurs these map to bovine chromosome 4 and segregate with a frame-shift mutation (p.A56RfsX87) in *twist family bHLH transcription factor 1 (TWIST1)* gene (Capitan et al., 2011). No homozygous individuals could have been found, pointing towards a lethal factor, which is in accordance with embryonic lethality in *TWIST1* knock-out mice (Chen and Behringer, 1995).

2.2.2. Polyceraty

Polyceraty describes the occurrence of more than two horns. Up to six horns are reported (Porter et al., 2016). This trait is mainly found in sheep (Dýrmundsson, 2005), where some breeds, like Jacob sheep (Porter et al., 2016), carry multiple horns as signature trait. Polycerate goats are described as well (Giovanoli, 1919; Herrera et al., 2007). In both species a link to eyelid deformity is described (Herrera et al., 2007; Henson, 1981; Gascoigne et al., 2017). How and why polyceraty evolved remains unknown, but recently two slightly different variants (Table 2), in the *homeobox D1* (*HOXD1)* gene on ovine chromosome 2 and caprine chromosome 2 were found to segregate with the trait in sheep and goats, respectively (Allais-Bonnet et al., 2021) confirming first mapping trials (Greyvenstein et al., 2016; He et al., 2016; Kijas et al., 2016). No information is available about the occurrence of multihornedness in cattle.

Table 2: Details on the variant segregating with polyceraty in sheep and goats.(modified after Allais-Bonnet et al., 2021; Nicholas and Tammen, 1995)

Species	Chromosome	Gene	Variant	Location	Mode of inheritance	Reference
Sheep (*ovis aries*)	2	*HOXD1*	Deletion, 4 bp	Oar_rambouillet_v1.0: NC_056055.1 (133949709..133947471)	Autosomal co-dominant	Allais-Bonnet et al. 2021
Goat (*capra hircus*)	2	*HOXD1*	Delins, 137 kb	ARS1: NC_030809.1 (115593830..115596023)	Autosomal dominant	Allais-Bonnet et al. 2021

2.3. Differences in husbandry management for horned animals

Reviewing recommendations for commercial goat housing and management Zobel et al. (2019) stated that the available information is limited and a great proportion is rather based on practical experiences than on science-based research (Zobel et al., 2019).

Horns in goats are used in offense and defense (Geist, 1960) for various resources, including sexual partners and food (Shi and Dunbar, 2006; Shank, 1972; Geist, 1966; Lundrigan, 1996; Stankowich and Caro, 2009). Goats have a complex social structure and constant group formation of relatively small core groups (~ 12 individuals Stanley and Dunbar, 2013), with a strict hierarchy (Zobel et al., 2019). Groups are formed and structured by rank fights (agonistic behavior) and affiliative behavior (Zobel et al., 2019). It was shown that there is a higher tendency for injuries regarding the udder in horned compared to polled flocks (Waiblinger et al., 2010). Horns do not only bear a risk for flockmates but also for stockpersons to become physically injured (Knierim et al., 2015; Braun et al., 2016; Goldblum et al., 1999; Katsos et al., 2019; Tijjani et al., 2015). However, injuries caused by horn-induced bruises can also cause economic damage, for example by reducing milk yield or by lowering the meat quality of valuable carcass cuts (Mendonça et al., 2016; Youngers et al., 2017; Collins and Huey, 2014). In intensive-housing systems, animals cannot always avoid flockmates and maintain individual distance, potentially leading to social conflicts and horn use (Aschwanden et al., 2008). Therefore, the named risk factors need to be considered in housing and management of horned livestock and often require adaptations. To give more space for evasion measures like a lower stocking density, the offer of a raised level (Zobel et al., 2019) and additional feeding space (Loretz et al., 2004; Waiblinger et al., 2010) in combination with modified feeding rails (Aschwanden et al., 2009; Hillmann et al., 2014; Waiblinger et al., 2010) are advised. In addition, a stable herd/flock structure, e.g. by a longer service life, is recommended as a measure to avoid social stress and associated combative interactions (Waiblinger et al., 2010). Waiblinger et al. (2010) stated that named improvements of housing and general management increase animal welfare in goats in general, independent of the respective horn status.

It has also been shown that management and the housing environment, in particular the availability of space, are decisive factors in the successful keeping of horned cows (Menke et al., 1999; Waiblinger et al., 2001).

For sheep no recommendations are found in the literature.

2.4. Dehorning of small ruminants

To account for the risk factors associated with horns described in chapter 2.3. disbudding is a routine management practice in cattle and goats husbandry in many countries worldwide (Boyd, 1988; Cozzi et al., 2015).

Even though several countries and the European Union (EU) defined animal welfare principals (e.g. European Union, 1998, 2008)) which include the avoidance of pain induced by management procedures like for example disbudding it is often still seen as a routine measurement, due to legal exceptions.

In addition to legal restrictions that aim at avoiding pain caused by interventions on the animal, also functional properties of horns, e.g. in thermoregulation (Parés-Casanova and Caballero, 2014) are discussed as arguments against dehorning.

Disbudding of sheep is not a common practice, as many commercially used breeds are bred genetically polled.

2.4.1. Methods, risks and regulation in Germany

Three common methods of disbudding are cautery, cryosurgical and caustic paste (Bengtsson et al., 1996; Hempstead et al., 2018b; Vickers et al., 2005). According to a survey in European countries cautery method using an hot iron was preferred in disbudding calves (Gottardo et al., 2011; Staněk et al., 2018) while in the U.S. state Wisconsin caustic paste was the primary method (Saraceni et al., 2021) . All named methods cause pain to the treated animals (Heinrich et al., 2010; Allen et al., 2013; Stafford and Mellor, 2005; Waiblinger et al., 2010), whereby caustic paste and cryosurgical disbudding appeared to cause greater acute pain in goat kids when compared to the cautery method (Hempstead et al., 2018a). In addition to the animal welfare aspect, economic reasons can also be evoked for the use of analgesics. Investigations have shown that pain can lead to reduced feed intake and thus reduced daily weight gains (Bates et al., 2015; Borderas et al., 2009). Cortisol concentration, expressing pain and stress, was even elevated in animals in which dehorning was performed under local anesthesia, showing that a combination with analgesics is important in goat kids (Alvarez et al., 2009) and calves (Stock et al., 2013). This contradicts earlier results in calves, where a reduced pain sensitivity was observed after dehorning under local anesthesia (Graf and Senn, 1999).

Nevertheless, especially in goats, disbudding-related injuries occur often. Especially cautery disbudding, if performed incorrectly, has a high potential of skull damage, leading to thermal injuries of the brain (Hempstead et al., 2018a; Sanford, 1989; Waiblinger et al., 2010), being still considered the most effective method compared to others (Still Brooks et al., 2021). A large proportion (> 50%) of the observed brain injuries in goats in a retrospective study could be diagnosed as suppurative inflammation due to, among other things, injuries from dehorning (Allen et al., 2013). This confirms findings from a New Zealand working group showing a high risk of brain injury from thermal disbudding of neonatal kids (Thompson et al., 2005). Another factor that enlarges the risk of injuries is that most veterinarians will only proceed disbudding of goat kids irregularly and therefore lacking on experience/practice (Clayton, 2013). To address this factor, in 2008 a system has been established in Switzerland where only certified farmers are allowed to perform the mandatory anesthesia and dehorning of their own kid goats independently – “anesthesia delegation model (ADM)” (Alsaaod et al., 2014; Wagmann et al., 2018). Due to Wagmann et al. (2018) the success of this program is questionable as in over one third of the analyzed cases the anesthesia was inadequate. Another reported issue with improper disbudding is the remaining of horns or parts of them, causing pain as well (Battini et al., 2014).

In Germany, the legal situation differs significantly from other countries, even though the painful intervention has also been the subject of criticism elsewhere for a long time. Referring to paragraphs §5 and §6 of the German Animal Welfare Act, the prohibition of avoidable pain and suffering, the dehorning of sheep, goats and cattle is prohibited as a routine measure in

Germany (Deutscher Bundestag, 2006). However, there is currently an exception for the latter (§ 5 (3)2. and §6 (1)3.), allowing calves younger than six weeks to be dehorned even without the use of anesthesia (Deutscher Bundestag, 2006).

2.5. Breeding for polledness

Taking animal welfare concerns and the legislation into account the need for an alternative of dehorning becomes obvious. As previously described, polledness occurs naturally in sheep, goats and cattle and therefore the basics for breeding for polledness in those species are present. In some cases genetic testing for polledness is available to support breeders with early information about an individual's genotype (Randhawa et al., 2020). Nevertheless, this approach has limitations, as described below.

The first one to be mentioned is the risk of inbreeding, accompanied by the loss of genetic diversity, when trying to push the trait of polledness into a mainly horned population (Schafberg and Swalve, 2015; Windig et al., 2015; Scheper et al., 2016). Spurlock et al. (2014) described the risk of losing genetic merit which is associated with the selection for the polled trait in cattle (Spurlock et al., 2014).

Another risk, which is especially important for goats, is that polledness can be linked to undesired traits or even defects.

2.5.1. Interaction of horn status and health-related characteristics

Horn status, in peculiar polledness, is often thought to have a negative influence on performance parameters in livestock. Such adverse associations are difficult to prove scientifically, as performance is influenced multifactorially (Scheper et al., 2021; Cozzi et al., 2015; Goonewardene et al., 1999). Nevertheless, there are also known characteristics of varying impact on the affected animal, whose association with the horn status is easier to prove. Some of the described characteristics associated with polledness are also known to affect only individual families.

2.5.1.1. Polled Intersex Syndrome (PIS)

PIS is the most commonly known constraint in farm animals that is related to the horn status. The phenomenon was first described in 1944 when an unusual sex ratio, increased number of animals with male habit, was observed in polled goat flocks (Asdell, 1944). It became clear that, in contrast to other horn-bearing species, there is a connection between the absence of horns and disorders in sex development in domestic goats. Particular effects are seen in females (XX), which are affected by phenotypically variable intersexuality when homozygous polled (Pannetier et al., 2012; Just et al., 1994; Vaiman et al., 1997). The phenotypic variability complicates the early detection of affected goats (Szatkowska et al., 2014). While polledness is known to be dominant inherited, the associated intersexuality is a recessive trait. Infertility or lower fertility in male PIS-affected goats may occur, but the exact circumstances and impacts have not yet been discovered (Pannetier et al., 2005). An association between PIS-affection and various growth traits could be ruled out, at least for Guanzhong dairy goats (Zhang et al., 2020). The locus for PIS was mapped to chromosome one (Vaiman et al., 1997) and narrowed down to a 11.7-kb sized deletion in the region of *PIS-regulated transcript 1*

(*PISRT1*) and *forkhead box L2* (*FOXL2)* gene, affecting both genes transcription (Pailhoux et al., 2001). The influence of *FOXL2* on sex determination and regulation in horn bud differentiation was confirmed in further studies (Allais-Bonnet et al., 2013; Boulanger et al., 2014). For example, loss of function of *FOXL2* has been shown to lead to female-to-male sex reversal (Boulanger et al., 2014). A genome wide association study confirmed the association of the region previously published with PIS. Furthermore, evidence was found for a single genetic, but more complex basis then suggested for PIS in European and non-European breeds (Kijas et al., 2013). The identification of affected animals, early in life was considered particularly important for the agricultural livestock production but so far there are only attempts realized that focus on sex determination in polled goats. For example via a simple PCR detection of X- and Y-specific variants in the *amelogenin* gene, focusing on the detection of XX-males (Fábián et al., 2017).

2.5.1.1.1. Other causes for intersexuality in livestock

The already described phenomenon of PIS is not the only known cause of intersexuality in goats. And also for other farm animals the condition of intersexuality is not rare – various causes have been described.

One well-known cause of intersexuality is freemartinism (Padula, 2005; Nicholas and Tammen, 1995), mainly occurring in mixed-gender twin gestation in cattle (OMIA 000393-9913), but cases in sheep (OMIA 000393-9940) and goats (Szatkowska et al., 2014) are reported as well. Due to anastomosis in early gestation, masculinizing hormones (anti-mullerian hormone and testosterone) and hematopoietic stem cells (HSC) are transferred from the male to the female embryo in heterosexual twins, causing the female to be born infertile (XX/XY chimerism) in most of the cases (Padula, 2005). This is possible because the male gonad differentiation begins earlier in gestation then in females and gets active with steroid production (Short and Bulaban, 1994).

In addition, a number of gene variants are known to be associated with disorders of sexual development (DSD) in humans and mice (Larson et al., 2012; Eggers and Sinclair, 2012) which might also be involved in similar cases in livestock. Yang et al. (2021) showed that altered expression of some of those genes where detectable in intersex goats as well. Thereby they found that in the expression of *WT1 transcription factor* (*WT1), doublesex and mab-3 related transcription factor 1* (*DMRT1*), *GATA binding protein 4* (*GATA4*), *Anti-Mullerian hormone (AMH)*, and *desert hedgehog (DHH)* genes differed between testicular (male habit) and ovarian (female habit) types of intersexes (Yang et al., 2021).

2.5.1.2. Abnormalities of the brows and eyelids

In polled cattle, a link between polledness and a specific eyelash-and-eyelid phenotype is described. A supernumerary row of lashes on the inner part of the eyelid is reported in polled animals, as well as an eyelid hypertrichosis (extensive hair growth) (Allais-Bonnet et al., 2013).

In sheep and goats, abnormalities of eyebrows and eyelids have also been described, not in connection with polledness, but in the presence of an increased number of horns (polyceraty) (Allais-Bonnet et al., 2021; Lühken and Drögemüller, 2021). The congenital split upper eyelid defect (SUED) for example is primarily found in polyceratous sheep and was first described in Jacob sheep (Henson, 1981). Little is known on the effects on animal welfare, for example due to the severity of damage to the cornea surface, or performance parameters of affected individuals (Gascoigne et al., 2017). A case report on goat flocks in Extremadura, Spain, showed that the association also exists in multiple-horned goats (Herrera et al., 2007). It is not yet known whether the variants in the *HOXD1* gene recently identified as causal for multihornedness in sheep and goats are also associated with the occurrence of these eyelid anomalies (Allais-Bonnet et al., 2021).

2.5.1.3. Polled and Multisystemic Syndrome (PMS)

The PMS was just described in the polled progeny of a single Charolais sire (Capitan et al., 2011). A number of other symptoms could be observed in the affected offspring. These included among others facial dysmorphism, variable neurological disorders, chronic diarrhea and anomalies in reproduction with low progesterone levels. The latter one described for nine out of 14 PMS-affected females. Furthermore the unusual gender distribution in the offspring population points towards a male-specific lethal factor, inherited dominantly (Capitan et al., 2011).

A 3.7-Mb deletion on bovine chromosome two was found to be causative, for which the founder bull was a somatic mosaic. The variant affects two complete genes (*zinc finger E-box binding homeobox 2* (*ZEB2*) and *glycosyltransferase like domain containing 1* (*GTDC1*)) and a part of the *Rho GTPase activating protein 15* (*ARHGAB15*) gene (Capitan et al., 2011). Loss of *ZEB2* is also known in humans, affected individuals have Mowat-Wilson syndrome (MOWS or MWS), which has symptomatic similarities to PMS (Birkhoff et al., 2021; Mowat et al., 1998).

2.5.1.4. Abnormal skull shape, small body size and subfertility in Fleckvieh cattle

Another condition, which was found in a polled Fleckvieh cattle bull and its progeny is as well segregating with a mutation in the second exon of *ZEB2* (Gehrke et al., 2020a). The described polled condition is in association with a deformed skull, reduced height and subfertility, whereby also here a connection to the MOWS was drawn. A de novo 11-bp deletion in *ZEB2* gene was found to be causative, and was first detectable in the affected polled Fleckvieh cattle bull, that was born to horned parents, which led to the exclusion from the classic dominant hornless variants (Gehrke et al., 2020a).

2.5.1.5. Type 2 Scurs Syndrome

The autosomal dominant inherited type 2 scurs, just present in Charolais cattle, that segregate with a frameshift mutation in the *TWIST1* gene (bovine chromosome 4), differ from the classical scurs (Capitan et al., 2011). What distinguishes type 2 scurs from classic ones is that in addition to horn abnormalities (loosely attached small or deformed appendices), skull interfrontal suture synostosis is also present. An elongated protrusion on the forehead is visible in affected animals, getting more prominent with age. It is considered that the condition has no severe consequences in heterozygous state. However, as no individual was observed that carries the underlying variant in a homozygous state, it is presumed to act as a lethal factor (Capitan et al., 2011).

2.5.2. Genetic engineering

A promising and relatively new approach to advance the breeding for polledness, especially in those species where polledness is associated with additional undesirable traits, is the use of genetic engineering.

Public and research focus was drawn on these techniques when in 2012 the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 system, originally found in adaptive immunity in bacteria, was published, highlighting its potential for programmable genome editing (Jinek et al. 2012). Nevertheless, already before that attempts were made, editing of polled cattle using so called transcription activator-like effector nucleases (TALENs), was achieved (Tan et al., 2013).

The result-oriented evaluation of genetic engineering by means of gene scissors led to a classification as classical genetic engineering by the European Court of Justice and was groundbreaking in the European Union (EuGH, 2018). Products produced in this way are therefore subject to the strict guidelines for genetically modified organisms, which regulate their release (European Parliament and the Council, 2001). The judgement tried to reflect the average public opinion. The public is highly skeptical of genetically modified foods, with country-specific differences in attitude (Frewer et al., 2013; Canavari and Nayga, 2009).

Nevertheless, the methodology is applied, also in the field of farm animals. Especially polledness in cattle was already focused (Carlson et al., 2016), while it was not applied for polledness in small ruminants yet.

2.6. Aim of this study

The aim of this work was to address genetic polledness in small ruminants, since the knowledge here is only rudimentary compared to the scientific knowledge for the same trait in cattle. This is despite the fact that the trait is associated with severe breeding impairments in goats due to the polled intersex syndrome (PIS). In this area, the aim of the work was to establish a practical genetic test for early detection of affected animals and thereby facilitate practical breeding for polledness in goats. Since the trait polledness in bovid species is highly diverse in many aspects, including the genetic basis, there is no review so far that compares this trait in the most important commercially used species cattle, sheep and goats. In order to provide such an up-to-date overview and comparison, the preparation of a review was another important goal of this work. The collection of current research results on the inheritance of horn-related traits shows that, especially in sheep, only little across-breed knowledge has been generated in recent years. However, many aspects showed a certain breed dependency. In order to lay the foundation for further research in this field, another aim of this work was to verify known variants and markers for traits related to horn status in the breed Icelandic sheep, as this breed is diverse, highly isolated and has never been analyzed for horn-related traits on the genetic basis in detail.

3. ORIGINAL WORKS

3.1. First publication

Simon, R.; Lischer, H. E. L.; Pieńkowska-Schelling, A.; Keller, I.; Häfliger, I. M.; Letko, A.; Schelling, C.; Lühken, G.; Drögemüller, C. (2020): New genomic features of the polled intersex syndrome variant in goats unraveled by long-read whole-genome sequencing. In: Animal Genetics 51 (3). DOI: 10.1111/age.12918.

- Parts of this publication were presented as poster and talk at the 37th conference of the International Society for Animal Genetics (ISAG) 2019 and awarded a poster prize and a travel bursary. Additionally parts of this publication were presented as talk at the European Molecular Biology Laboratory (EMBL) Symposium: The Molecular Basis and Evaluation of Sexual Dimorphism (2020) and at the International Congress on the Breeding of Sheep and Goats (2020).
- Contribution in: investigation, writing – original draft preparation, writing – review and editing, visualization

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New genomic features of the polled intersex syndrome variant in goats unraveled by long-read whole-genome sequencing

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Summary

In domestic goats, the polled intersex syndrome (PIS) refers to XX female-to-male sex reversal associated with the absence of horn growth (polled). The causal variant was previously reported as a 11.7 kb deletion at approximately 129 Mb on chromosome 1 that affects the transcription of both *FOXL2* and several long non-coding RNAs. In the meantime the presence of different versions of the PIS deletion was postulated and trials to establish genetic testing with the existing molecular genetic information failed. Therefore, we revisited this variant by long-read whole-genome sequencing of two genetically female (XX) goats, a PIS-affected and a horned control. This revealed the presence of a more complex structural variant consisting of a deletion with a total length of 10 159 bp and an inversely inserted approximately 480 kb-sized duplicated segment of a region located approximately 21 Mb further downstream on chromosome 1 containing two genes, *KCNJ15* and *ERG*. Publicly available short-read whole-genome sequencing data, Sanger sequencing of the breakpoints and FISH using BAC clones corresponding to both involved genome regions confirmed this structural variant. A diagnostic PCR was developed for simultaneous genotyping of carriers for this variant and determination of their genetic sex. We showed that the variant allele was present in all 334 genotyped polled goats of diverse breeds and that all analyzed 15 PIS-affected XX goats were homozygous. Our findings enable for the first time a precise genetic diagnosis for polledness and PIS in goats and add a further genomic feature to the complexity of the PIS phenomenon.

Keywords *Capra hircus*, copy number variant, FISH, horn, long-read sequencing, structural variant, gene testing, precision medicine

Introduction

In 1944, the British reproductive biologist Sydney Asdell reported that all of the intersexual goats he had seen were hornless (polled) (Asdell 1944). In contrast to other ruminant species, the trait of polledness is connected with disorders of sexual development in domestic goats. Intersexuality in goat is a recessive trait affecting exclusively genetically female (XX) individuals and is completely associated with the dominant mutation for the absence of horns in males and females (OMIA 000483-9925). The so-called polled intersex syndrome (PIS) is characterized by homozygous polled XX individuals which are infertile owing to diverse intersexual phenotypes (Fig. 1; Pannetier *et al.* 2012). Besides its sex-reversing effect on XX individuals, the PIS mutation can also be responsible for infertility in homozygous polled XY individuals, resulting apparently from mechanical obstruction of the epididymis (Pailhoux *et al.* 2005).

The existence of a visible marker (absence of horns) made goats an interesting model for identifying sex-determining genes in mammals (Pailhoux *et al.* 2005). In 2001, a 11.7 kb non-coding deletion located approximately 200 kb upstream of the *FOXL2* gene was detected as a PIS-associated genomic variant (Pailhoux *et al.* 2001). Initially,

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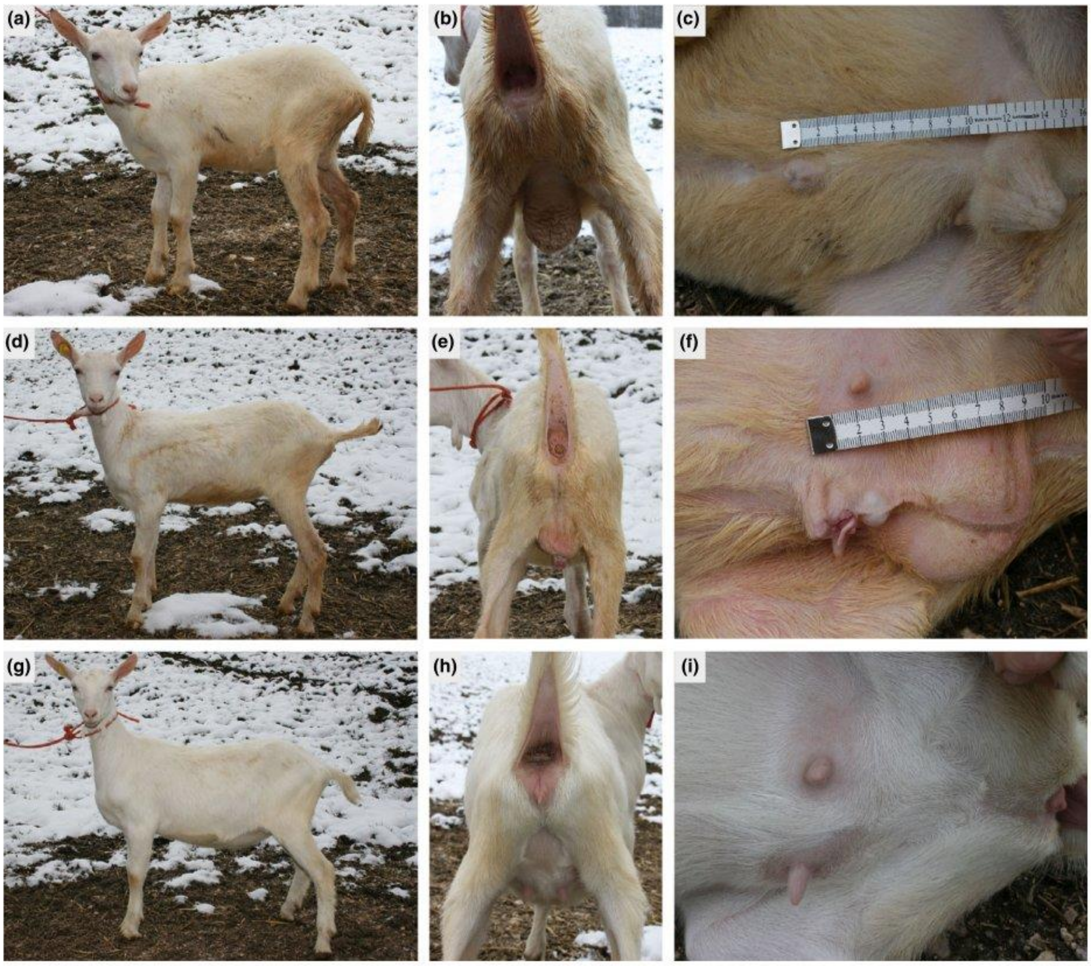

Figure 1 The polled intersex syndrome (PIS) in goats. Appearance of the external genitalia in three hornless Saanen goats: a heterozygous polled genetically male (XY) animal (a–c), a PIS-affected homozygous polled XX male (d–f) and a heterozygous polled genetically female (XX) animal (g–i). Note the masculinized intersex phenotype of the PIS-affected goat.

the origin of sex reversal in XX goats homozygous for the PIS variant was unclear because of the complexity of the mutation that affects the transcription of both *FOXL2* and several lncRNAs (Pannetier *et al.* 2012). Based on these findings, alternative models for the sex-determination process in goats in comparison with mice were proposed (Elzaiat *et al.* 2014). In 2014, it was shown by genome editing that *FOXL2* loss of function dissociated from loss of lncRNA expression is sufficient to cause female-to-male sex reversal in XX goats (Boulanger *et al.* 2014).

The phenotypic identification of PIS cases is challenging. Especially as some XX intersexual polled goats cannot be distinguished by their phenotype from normal polled males (XY) before puberty, it would be valuable to have a simple test diagnosing both the genetic sex and the genotype for PIS in newborn animals (Fábián *et al.* 2017). Our trials to establish genetic testing for PIS with the existing molecular genetic information failed owing to repetitive sequences on both ends of the deletion. Recently, a first PCR-based method for the detection of the PIS-associated 11.7 kb deletion was published (Zhang *et al.* 2019). In the light of two studies speculating that the reported deletion was not complete but partial (Li *et al.* 2011), or that maybe different versions of the PIS deletion exist (Kijas et al. 2013), we decided to revisit the genomic details of the PIS-associated genetic variant using long-read whole-genome sequencing.

Structural variants (SVs) like the reported PIS-associated deletion are often poorly assayed using currently dominant short-read sequencing technologies but can be detected using recently established long-read sequencing

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technologies from Pacific Biosciences and Oxford Nanopore Technologies (De Coster & Van Broeckhoven 2019a, Mantere *et al.* 2019). Although these sequencing technologies have a lower single nucleotide accuracy of approximately 85–90%, they have the advantage of a better mappability in repetitive regions, further extending the part of the genome in which variation can be reliably called (De Coster *et al.* 2019b). Recent results corroborate that complex SVs cause a significant number of Mendelian traits and that precise resolution of breakpoints can be achieved by long-read genome sequencing (Sanchis-Juan *et al.* 2018; Lappalainen *et al.* 2019). A first successful application of long-read whole-genome sequencing in domestic animal genetics confirmed a previously reported quadruplication as a causal mutation for the belted phenotype in cattle (Rothammer *et al.* 2018).

Herein we report the identification of a complex structural variant associated with caprine PIS that enables for the first time robust genetic testing and adds a previously unknown detail to understand the complexity of the PIS phenotype. This study represents the second successful application of long-read whole-genome sequencing technology to unravel the causative mutation underlying a Mendelian trait in a domestic animal species.

Material and methods

Animals

This study included 814 samples (152 XY, 662 XX) from animals of 23 different goat breeds (Table S1). The dataset consisted of 334 polled (hornless) goats, including 15 suspected as PIS (XX males), and 480 horned animals. For some of the polled animals, the genotype for polled (homozygous or heterozygous) was derived from the horn status of their progeny. Genomic DNA was extracted from EDTA-stabilized blood or hair roots using routine methods.

Long-read whole-genome sequencing

We performed long-read whole-genome sequencing of two individual goats: a polled PIS-affected XX Saanen goat (SAN096) and a horned XX Valais Blacknecked goat (VAG203) that was initially sequenced for the purpose of another ongoing project in our laboratory. Genomic DNA of these two individuals was isolated from blood using the DNA MagAttract HMW DNA Kit (Qiagen). DNA quality was assessed by running 1 µl on the Femto Pulse automated pulsed-field capillary electrophoresis system (Agilent Technologies) to ensure a significant fraction of high-molecular-weight genomic DNA. DNA concentration was assessed using the dsDNA HS assay on a Qubit fluorometer (ThermoFisher Scientific). We prepared seven individual fragment libraries per animal using 5 µg caprine genomic DNA as starting material according to the SQK-LSK108 1D Genomic DNA by Ligation Kit (Oxford Nanopore Technologies) without performing the optional DNA fragmentation step. MinION sequencing was performed as per the manufacturer's guideline using R9.4 flow cells. MinION sequencing was controlled using Oxford Nanopore Technologies MinKNOW software. Base calling was performed using Albacore (https://github.com/dvera/albacore). We collected 6 572 301 (SAN096) and 5 068 569 (VAG203) reads, which corresponds to roughly 14× and 9× coverage owing to the different average read length between the two goats (3991 bp for SAN096 vs 8079 bp for VAG203; Table S2). The reads were mapped against the goat reference genome assembly using MINIMAP2 version 2.8 (Li 2018). All genome positions refer to the ARS1 reference sequence assembly (Bickhart *et al.* 2017). The INTEGRATIVE GENOME VIEWER software (Thorvaldsdóttir *et al.* 2013) was used for visual inspection to identify structural variants in the critical regions at approximately 129 and 150 Mb on chromosome 1.

Short-read whole-genome sequencing

For comparison with short-read whole-genome sequencing data based on PCR-free DNA libraries sequenced for 2 × 150 bp paired-end reads on Illumina HiSeq instruments, we used 20 publicly available control genomes with an average sequence depth of 15× (Table S3), which were produced during other ongoing projects of our group as described previously (Becker *et al.* 2015; Reber *et al.* 2015; Menzi *et al.* 2016). This cohort of genomes included two homozygous polled males (XY), five heterozygous polled females (XX) and 13 horned goats of both chromosomal sexes belonging to 10 different breeds (Table S3). Coverage for the two regions of interest was calculated by counting the read depth of each base in the defined regions of interest using SAMTOOLS version 1.3 (Li *et al.* 2009). The average read depth per base for each of the three genotypes (horned, heterozygous, and homozygous polled) was used to create the circos plot using OMICCIRCOS package (Hu *et al.* 2014).

Cytogenetic analyses

Heparinized blood was collected from three goats (horned, heterozygous and homozygous polled) and chromosome preparations were obtained after short-term lymphocyte cultures according to a modified protocol of Arakaki & Sparkes (1963). The modifications consisted of a longer treatment with colcemide (2 h) and pre-cooling the fixative at −30°C. Based on the whole-genome sequencing results, two BAC clones were selected for FISH mapping: the caprine clone 376H9 containing the PIS-associated deletion at approximately 129 Mb (Schibler *et al.* 2000; Pailhoux *et al.* 2001) and the clone CH243-464K19 selected from the sheep BAC library (Ratnakumar *et al.* 2010), corresponding to the approximately 150 Mb region. The BAC clone

CH243-464K19 was identified by BLASTN searches of the goat chromosome 1 sequence to ovine BAC end sequences and obtained from the BACPAC Resources Center (https://bacpacresources.org). The well-characterized and easy to search sheep BAC library was used instead of searching for a suitable goat BAC clone owing to the lack of accessibility. The insert sequence of 141 kb includes parts of the ovine *ERG* gene and corresponds to goat chromosome 1 from 150 659 310 to 150 800 390.

The BAC DNA was prepared using the Qiagen Midi plasmid kit according to the modified protocol for BAC clones (Qiagen). The BAC DNA was labeled by nick translation using FITC (clone 376H9) or Texas Red (CH243-464K19) (Rigby *et al.* 1977). Double FISH was performed on metaphase chromosome spreads following a published protocol (Pieńkowska-Schelling *et al.* 2008). After FISH, identification of goat chromosomes (Cribiu *et al.* 2001) was performed using DAPI-banding by pipetting 25 μl Vectashield H-1200 (1.5 μg/ml DAPI in antifade mounting medium; Vector Laboratories) onto the slides.

PCR and targeted genotyping

To characterize the breakpoints of the identified variant, primers were designed using Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/) (Table S4). Two amplicons specific for the caprine PIS allele were successfully generated with the AmpliTaq Gold 360 Master Mix (Life Technologies). The breakpoints of the structural variant at approximately 129 and 150 Mb were verified by subsequent Sanger sequencing on an ABI 3730 capillary sequencer (Life Technologies).

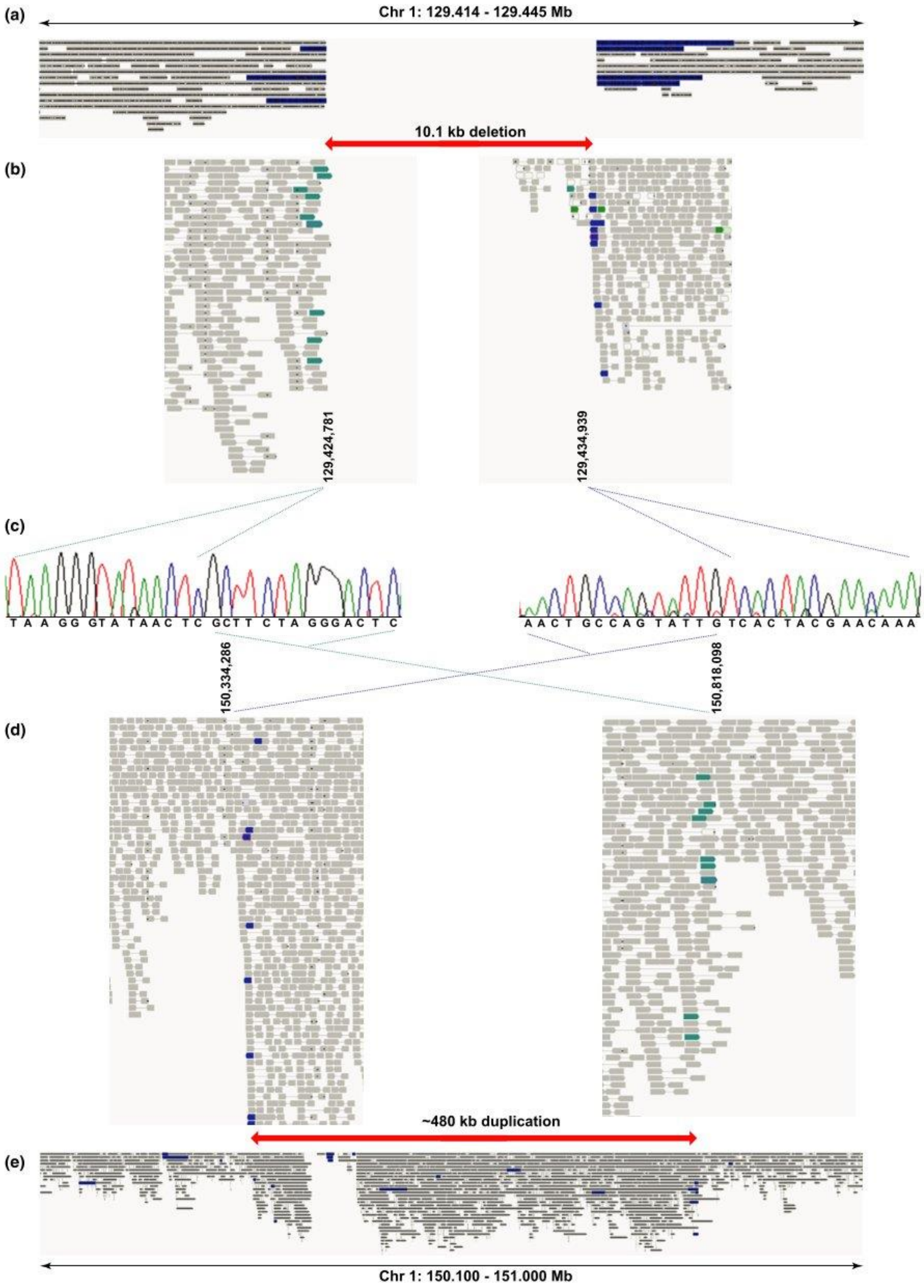
A multiplex PCR allowed the determination of the individual polled genotypes in combination with the determination of the genetic sex by fragment length analysis. An 143 bp amplicon detecting the variant allele and a second 398 bp product detecting the wt allele in the region of the upstream breakpoint at 129.425 Mb were amplified simultaneously (Table S4). In parallel, three previously described primers targeting the X chromosomal *AMELX* and the Y chromosomal *AMELY* genes (Tsai et al. 2011) were added to this multiplex PCR using the QIAGEN Multiplex PCR Master Mix for amplification (Table S4).

Results and discussion

Although the fascinating question of gene variation responsible for the XX female-to-male sex reversal observed in homozygous hornless (polled) goats was answered nearly two decades ago by Pailhoux *et al.* (2001), so far no simple tool has been developed to genotype animals in question for PIS. The reported 11.7 kb deletion was characterized by interspersed repeat elements in the breakpoint regions and it has been speculated that this variant has not been fully described (Li *et al.* 2011; Kijas et al. 2013). Recently a newly described *de novo* goat genome sequence became available (Bickhart *et al.* 2017). It was reported to be the most contiguous diploid vertebrate assembly generated thus far using whole-genome assembly and scaffolding method (Worley 2017). Therefore, we decided to revisit the published PIS-associated deletion on goat chromosome 1 by applying new long-read whole-genome sequencing technology to determine the precise genomic architecture of the causative mutation.

Focusing on the chromosome 1 critical interval at approximately 129 Mb, we analyzed the long-read whole-genome sequence data of two genetically female (XX) goats, a PIS-affected homozygous polled animal in comparison with a horned control. The ASR1 goat assembly is based on the genome of a horned San Clemente goat (Bickhart *et al.* 2017). Thus it could be assumed that the variant causing polledness was not present in the reference assembly. We inspected the mapped genome data of the two sequenced animals and specifically searched for structural variants by visual inspection of sequence reads mapping to the previously reported genomic region at approximately 129 Mb on chromosome 1 (Pailhoux *et al.* 2001). This indicated the presence of a significantly shorter deletion of only 10.1 kb in size in the PIS genome, compared with the published 11.7 kb-sized deletion (Fig. 2a). In the sequenced horned control this region showed a sequence coverage that was similar to the average genome-wide coverage (Fig. S1a). Interestingly, we observed *in silico* evidence for a homozygous duplication of approximately 480 kb about 21 Mb further downstream at approximately 150 Mb on chromosome 1 (Fig. 2e) in the PIS-affected XX goat. This second region was also normally covered in the genome of the

Figure 2 Genomic features of the PIS-associated variant on chromosome 1 in homozygous polled goats. (a) INTEGRATIVE GENOME VIEWER (IGV) screenshots of Nanopore long-read sequences data indicate a homozygous 10.1 kb deletion at approximately 129 Mb (red arrow). Seven reads displayed in blue support the presence of an insertion owing to split read mapping spanning the breakpoints. (b) Close-ups of IGV screenshots of Illumina short-read sequences illustrate the deletion and show discordant paired-end sequence reads aligning on two different segments of chromosome 1 at the boundaries of the variant sites (indicated in turquoise and blue). (c) Experimental confirmation of the complex structural variant. Sanger sequencing of PCR products of the variant allele precisely defines the breakpoints of the fusion of both chromosome 1 segments. IGV screenshots of Illumina short-read sequences (d) and nanopore long-read sequences (e) illustrate an approximately 2-fold increase across a approximately 480 kb segment indicating a homozygous duplication at approximately 150 Mb (red arrow). Apparently, the duplicated copy appears to be inversely inserted at the breakpoints of the deletion at approximately 129 Mb supported by several chimeric short-read pairs and long reads that partially map at a different location spanning the breakpoints.


(a)
Chr 1: 129.414 - 129.445 Mb
10.1 kb deletion
(b)
129,424,781
129,434,939
(c)
T A A GG GT A T A A C T C GCT T C T A GGG A C T C
A A C T G C C A G T A T T G T C A C T A C G A A C A A A
150,334,286
150,818,098
(d)
~480 kb duplication
(e)
Chr 1: 150.100 - 151.000 Mb

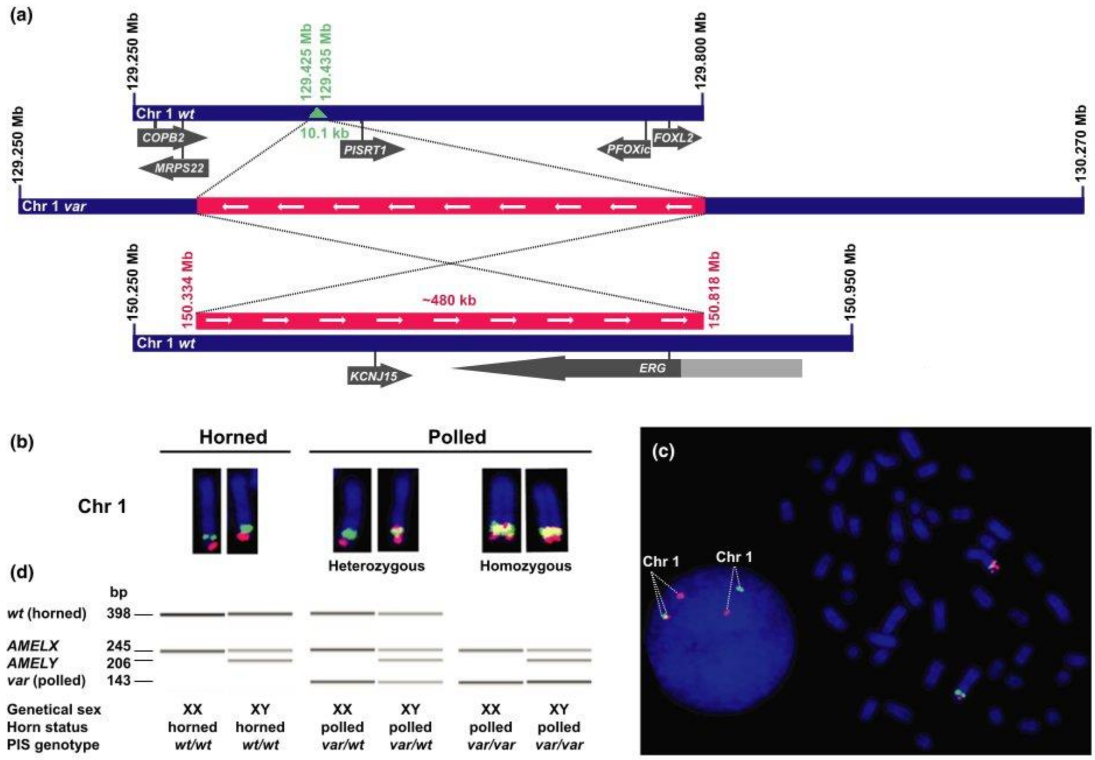


Figure 3 The PIS-associated complex structural variant on goat chromosome 1. (a) Schematic representation of the variant on chromosome 1 showing the inversely inserted approximately 480 kb segment (pink) replacing the deleted 10.1 kb segment (green). Location and orientation of the annotated genes and loci in both involved genome regions are shown (gray arrows). Note that the non-coding part of the ERG gene is not part of the duplication. (b) FISH experiment confirms the structural variant involving two different regions on chromosome 1. Hybridization of metaphases from horned and polled goats with a caprine BAC clone corresponding to the approximately 129 Mb region is revealed by green signals. An ovine BAC clone homologous to the approximately 150 Mb region is revealed by pink signals. Note the yellow signal indicating co-localization on chromosomes of polled animals. (c) FISH signals in an interphase nucleus and on metaphase chromosomes of a heterozygous polled goat. Note the normal pink and green signals on the wt chromosome and overlapping signals on the variant chromosome. (d) Electrophoresis-based fragment length analysis of a diagnostic multiplex PCR allows for the simultaneous discrimination of the six possible genotype combinations for genetic sex and PIS genotype.

sequenced horned control (Fig. S1a). Apparently, this second genome region is connected to the deleted segment as several sequence reads did not map continuously, but were split-mapped to these two different regions of chromosome 1 (Fig. 2; Fig. S1c). A detailed inspection showed several soft-clipped long-read sequences at the boundaries of the variant sites (Fig. S1b, d). A total of 15 reads were split-mapped across the breakpoint and this indicated that the duplicated copy of the approximately 480 kb segment appears to be inversely inserted at the upstream breakpoint of the 10.1 kb deletion (Fig. 3a).

In the course of other ongoing projects, the genomes of 20 goats from different breeds including seven polled goats (two homozygous and five heterozygous) were sequenced with short-read technology and inspected to evaluate our findings. The targeted analysis of the sequence coverage in the two involved regions of goat chromosome 1 confirmed the obviously shorter sized deletion at approximately 129 Mb present only in the polled individuals and absent in the horned animals, and an increased coverage across approximately 480 kb in the polled goats only at approximately 150 Mb (Fig. S2b). In the two homozygous polled animals, both the deletion and the duplication appeared homozygous, whereas the seven heterozygous polled goats showed one copy of the deleted part and three copies of the duplicated region. Interestingly, discordant paired-end short-reads aligning on the two different segments of chromosome 1 were noticed at the boundaries of the variant sites (Fig. 2b, d). The orientation of the chimeric read pairs in which both ends mapped to the same strand of chromosome 1 confirmed the previously observed inverse insertion of the larger (~480 kb) segment from the downstream region replacing the smaller (10.1 kb) deleted part (Fig. 2b, d).

To confirm these findings, we amplified and sequenced PCR products bridging the fusion points indicated by the discordant paired-end sequence reads. Sanger sequencing showed the precise breakpoints at 129 424 781 and 129 434 939 resulting in a deletion of 10 159 bp (Fig. 2c). The breakpoints of the previously published 11.7 kb deletion (GenBank accession no. AF404302) map to the region at 129 424 641 and 129 436 260, whereas the last 180 bp were not contained in the ASR1 assembly. Apparently, as the whole-genome region is characterized by interspersed repeat elements, the breakpoints have not been precisely determined before. In addition, sequence analysis placed the two breakpoints for the duplicated segment at 150 334 286 and 150 818 098 (Fig. 2c).

Subsequently, based on the identified breakpoint regions, two BAC clones were selected for FISH mapping to experimentally confirm these findings in goats with different horn phenotypes including a PIS-affected XX animal. In the horned goat this revealed signals at the two expected regions on chromosome 1 (Fig. 3b). In contrast, in the two polled goats the probe corresponding to the 150 Mb region showed split signals on either one or both copies of chromosome 1, confirming the complex structural variant observed before (Fig. 3b, c).

Intersexuality in the goat, studied for over a century (Petit 1894), brought to light new genes including *FOXL2*, involved in the female sex-determination pathway (Pailhoux *et al.* 2001). Already earlier it was postulated that a complete characterization of the PIS variant is needed before the role of PIS activity in other mammals or vertebrates can be elucidated (Vaiman & Pailhoux 2000). Our study adds so far unreported genomic details, making the PIS-causing variant more complex than believed before. Previously the understanding of the PIS mutation and its regulatory effects has been summarized in such a way that the PIS-deletion encompasses no coding regions and exerts transcriptional regulatory effects on at least three genes and long non-coding transcripts (Pannetier *et al.* 2012). It could be assumed that the detected insertion of an approximately 480 kb sequence into the region may have additional influence on the transcriptional regulation during embryonic development. This needs to be clarified in the future, in addition to the evaluation of whether the extra copy of the *KCNJ15* gene which is contained in the duplicated segment might play a significant role (Fig. 3a). *KCNJ15* encodes a potassium channel, which is implicated as an essential factor for the secretion of insulin, brain development, acid secretion in the lung and gastric acid secretion (Yuan *et al.* 2015). Without additional data about further functions of this gene, there is no reason to conclude that extra copies of that gene might have an impact on horn and/or gonad development. The same is true for the second annotated protein-coding gene (*ERG*), which is also partially contained in the duplicated segment (Fig. 3a). This oncogene for prostate cancer (Adamo & Ladomery 2016) is a member of the ETS (erythroblast transformation-specific) family of transcription factors that act as transcriptional regulators (OMIM 165080). Genes in the ETS family regulate embryonic development, cell proliferation, differentiation, angiogenesis, inflammation and apoptosis (Remy & Baltzinger 2000). Interestingly, besides the fact that ERG is important for vasculogenesis, angiogenesis and haematopoiesis, it is a crucial regulator of endocardial-mesenchymal transformation during cardiac valve morphogenesis (Vijayaraj *et al.* 2012). Furthermore, it was reported previously that ETS function is also critical in bone and cartilage development as it is expressed in developing mouse limb joints (Iwamoto *et al.* 2007). With its role in development that includes bone development, it could be speculated that the disturbance of regular horn growth in goats might be influenced by extra copies of *ERG*. Whether the additional non-coding sequence contains other important lncRNAs or affects *FOXL2* or *PISRT1* expression needs to be evaluated.

Finally, based on our findings, a simple diagnostic test was developed for straightforward genotyping of carriers for the identified variant in combination with the detection of the caprine sex chromosomes according to Tsai *et al.* (2011) (Fig. 3d). Meanwhile more than 800 goats of 23 different breeds, sex and horn status have successfully been tested (Table 1). All 15 suspected PIS-affected goats were confirmed genetically female (XX) and homozygous for the identified variant on chromosome 1. This contradicts the previous speculation that the basis of the intersex condition is more complex than simply the inheritance of two chromosomes carrying the PIS-associated variant (Kijas et al. 2013). Among the remaining 319 polled goats of 18 different breeds, the majority (310 animals) were heterozygous and only nine males (XY) without obvious changes in the sex organs or reproductive performance were tested as homozygous for the variant. This complex structural variant designated as *PIS* was absent in the 480 horned control genomes. These observations confirm previous results indicating that heterozygous hornless XX goats are fertile and that the PIS phenotype occurs only in XX homozygotes (Smith 1978; Pannetier *et al.* 2012). Altogether, although our genotyped cohort is not representative, we observed the described variant in all polled animals

Table 1 Genotype counts of the polled intersex syndrome (PIS)-associated variant in 23 different goat breeds.

			PIS genotype		
Phenotype	Genetic sex	Total	wt/wt	wt/var	var/var
Horned	XX	412	412		
	XY	68	68		
Polled	XX	250		235	15[1]
	XY	84		75	9
Total		814	480	310	24

[1] PIS-affected (XX males).

of various origins, which suggests that the mutation occurred before breed formation.

Intersexuality also occurs in horned goats, although it is extremely rare. We genotyped such an animal as well and verified the homozygous wt horn status (data not shown). We assume that this single case of intersexuality is most likely caused by other genetic factors, such as XX/XY chimerism, which has been reported earlier (Bongso *et al.* 1982; Batista *et al.* 2000).

In general, the obtained nanopore sequence reads of two goats varied in length from below 100 bp to 277 kb with an average of about 4–8 kb (Table S2). For both of the sequenced animals we obtained a significantly different amount of data although we used the same input of genomic DNA for the seven libraries produced for each sample. This resulted in different average genome coverages of about 9× and 14× (Table S2). Recently, a first exploration of human genome sequencing of two patients on the MinION sequencer at 11–16× depth of coverage showed that long reads, even with a relatively low coverage, are superior to short reads (average coverage of ~30×) with regard to detection of *de novo* structural variants (Cretu Stancu et al. 2017). These authors noticed a significant need to maintain DNA integrity during extraction and subsequent library preparation, which strongly influences the read length distribution. Moreover, they have identified a significant proportion of SVs that are not detected in short-read sequencing data of the same patient genomes. Other studies have demonstrated the potential benefits of long-read sequencing over standard short-read sequencing in clinical diagnostics to detect pathogenic SVs by identifying causative variants, which had remained undetected in previous analyses (Merker *et al.* 2018; Miao *et al.* 2018).

An obvious discrepancy with the reference explains the observed drop of coverage in an approximately 57 kb region within the approximately 480 kb-sized duplicated region in all sequenced animals, regardless of the horn status (Fig. S2b). A closer inspection of short-read alignments indicated a sharp breakpoint at position 150 446 881 (Fig. S2a), which also appeared in the long-read sequencing data (Fig. S1a). The mapped short reads in an approximately 57 kb segment upstream of this site showed a low mapping quality. Therefore, the exact dimension of the duplicated segment at approximately 150 Mb could not be determined. A *de novo* assembly of the long-read sequences would be helpful to resolve this genome region, which is characterized by repetitive sequence elements. As the available read depth of the two nanopore-sequenced goats is probably too low for this kind of analysis, we did not follow up at this time.

In conclusion, our study revealed a complex structural variant for caprine PIS. This work demonstrates the potential of long-read sequencing technology for animal genomics research by providing an example of the detection of a complex structural variant that had been missed before. We refined the complex nature of the detected PIS variant characterized by the fusion of a large duplicated chromosome 1 segment into the previously reported deleted part. This finding enables for the first time robust genetic testing and adds a previously unknown detail to understand the genetic complexity of the PIS phenotype. This study represents the second successful application of long-read whole-genome sequencing methods to unravel the causative variant underlying a Mendelian trait in a domestic animal species. The possibility for genome sequencing by nanopore sequencing or other long-read technology will facilitate such discoveries in the future, leading to further understanding of the role of structural variants in the genomes in general and in Mendelian disorders or traits in particular.

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Availability of data

Genome sequencing data were deposited in the European Nucleotide Archive (ENA, http://www.ebi.ac.uk/ena): the PIS-affected Saanen goat (sample accession no. SAMN09841857 within study accession no. PRJNA310684) and the control Valais Blacknecked goat (sample accession number no. SAMN09841858 within study accession no. PRJNA310684).

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Supporting information

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

Table S1 Horn status and PIS genotypes of 814 goats from 23 breeds.

Table S2 Output data of long-read whole-genome sequencing.

Table S3 Whole-genome short-read goat sequences.

Table S4 PCR primers for genotyping and determination of horn and sex status.

Figure S1 Nanopore long-read sequencing details.

Figure S2 Illumina short-read sequencing details.

3.2. Second publication

Simon, R.; Drögemüller, C.; Lühken, G. (2022): The complex and diverse genetic architecture of the absence of horns (polledness) in domestic ruminants, including goats and sheep. In: Genes 13 (5). DOI: 10.3390/genes13050832.

- Contribution in: conceptualization, formal analysis, writing—original draft preparation, writing—review and editing, visualization

genes

MDPI

Review

The Complex and Diverse Genetic Architecture of the Absence of Horns (Polledness) in Domestic Ruminants, including Goats and Sheep

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Abstract: Horns are the most obvious common feature of Bovidae. The naturally occurring absence of horns in these species, also known as polledness, is of surprisingly heterogeneous nature, although they are Mendelian traits. This review compares in detail the molecular differences among the causes of inherited polledness in the domestic ruminant species of cattle, yak, sheep, and goat based on the causal gene variants that have been discovered in recent years. The genetic causes for the lack of horns in small ruminants seem not only to be more complex, e.g., in sheep, breed-specific characteristics are still unexplained, but in goats, there is also the associated disorder of intersexuality—polled intersex syndrome (PIS). In connection with animal welfare and the associated discussion about a legal ban on the dehorning of all farm animals, naturally hornless animals and the causal genetic variants are of increasing research interest in the age of genome editing. However, the low acceptance of genetic engineering in livestock, especially in European societies, limits its use in food-producing animals. Therefore, genotype-based targeted selection of naturally occurring variants is still a widely used method for spreading this desired trait within and across populations, at least in cattle and sheep.

Keywords: horn development; hornless; intersexuality; Bovidae; bovine; caprine; ovine; ruminants; genome editing

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1. Horns in Bovid Species

During evolution, pecorans (i.e., higher ruminants) developed a notable diversity of bony skull attachments called "headgear", which are likely to have an identical genetic origin [1]. Ruminants are the only living group of mammals that have bony (osseous) headgear that is covered by a non-deciduous, unforked keratinous sheath [2]. Recently, comparative transcriptome analyses showed that bovine horns and cervid antlers share similar gene expression profiles and a common cellular basis that develops from neural crest stem cells [2]. Polyceraty, the presence of more than two horns, known in sheep and goat and observed since ~6000 BCE, is associated with defective HOXD1 function due to natural mutations [1].

Horns in bovids, the biological family of cloven-hoofed, ruminant mammals, including cattle, goat, and sheep, play a role in social behavior and protection. It is assumed that horns serve as a weapon in self-defense against predators and in ranking fights, for example, by stabilizing the head position during fights, as well as in sexual selection through intramale competition. Even impact absorption by horns is possible [3–7]. Horns can also be an attribute to the animal's social status and play a crucial role in mating success [8]. It has been shown that horns offer comparable advantages for females, as they do for males, in the competition for resources [9]. Furthermore, depending on their shape and size, horns can be used as tools in body care (Figure 1). In so-called "biodynamic agriculture", it is thought that the horns are important for the animal's thermoregulation or digestive processes [10,11].

Figure 1. Anglo Nubian goat scratching its back with the horn tip.

However, naturally occurring, genetically hornless (polled) animals are more or less common in most domesticated Bovidae, including various important livestock species (e.g., cattle, buffalo, yak, sheep, and goat) [12]. In general, hornlessness is apparently not associated with serious health restrictions. The reported fertility restrictions in hornless goats are an exception and will be discussed later. In addition, Stookey and Goonewardene (1995) showed that the polled condition in beef cattle bulls on performance testing stations had no disadvantages in the analyzed performance parameters compared to horned animals [13]. Furthermore, there is no evidence of a pleiotropic effect of the polled trait on the milk yield, fat content, somatic cell count as an indicator of mastitis, or female fertility in cows [14]. In the feral Soay sheep population on St. Kilda island, it has even been observed that horned rams have a higher annual breeding success but a shorter expectancy than scurred males, which have loosely attached horns with no bony connection [15].

2. Impact of Horn Status on the Welfare of Humans and Animals

Horned animals pose a danger when interacting with humans [16] and flock mates [17,18]. Bruising, which reduces meat quality [19], or serious injuries to the udder (Figure 2), which decreases the milking yield, can be the result of attacks on animals by horned individuals. The risks can be reduced, if possible, by adjusting the housing management [20,21] but cannot be eliminated completely. Therefore, many farmers prefer polled animals [22]. The disbudding of horned calves and goat kids is a painful standard husbandry procedure to reduce the described risks of injuries [22,23]. To address animal welfare concerns, the often-performed practice of dehorning is regulated by law in many countries. The European Council Directive 98/58/EC (last updated 2019), which states the minimum standards for the protection of farm animals, is the basis for the regulation of dehorning in the European Union [24]. EU member states have their own national agreements on the dehorning of livestock, which vary widely in stringency [25]. For example, in Germany, the Protection of Animals Act (Tierschutzgesetz, TierSchG) regularizes interventions on animals [26]. The physical removal of horns is generally prohibited unless there is a veterinary indication in an individual case (§6 TierSchG). There is an exception for calves younger than six weeks: dehorning without anesthesia is still allowed (§5 TierSchG), even if this condition is already in the focus of discussion and an animal-friendly alternative is demanded [27]. For organic farming, the EU legislation prohibits dehorning as a routine treatment, but local authorities can authorize exceptions [28]. Even though dehorning is partly legal, it is associated with suffering and pain for the animal [17,29]. Therefore, the need for and interest in genetically polled animals is increasingly apparent.

Figure 2. Fresh (in focus) and older, already crusty injury (at the base of the teat) on the left udder of a Saanen goat. The injuries were caused by horn blows from horned flock mates.

3. Diversity of Horn Status in Domestic Ruminants

There is a wide variety of naturally occurring forms of horn size, shape, and position, including rare forms of hornlessness (polledness) [30–33]. Breeds can have only one characteristic horn phenotype, i.e., be fully horned, such as Highland cattle and German Grey Heath sheep, or be hornless without exception, such as Aberdeen Angus cattle [12]. In general, the polled trait is more common in beef cattle than in dairy breeds. This man-made, breeding-induced differentiation can be explained by differences in animal husbandry and handling (e.g., temporary or permanent fixation vs. free-range with little or no restraint) [34]. In dairy cattle, for example, daily fixation and human contact during the lactation period are common. The reason that, in contrast to cattle and sheep, no completely polled goat breeds are known so far, will be explained later. On the other hand, there are numerous breeds in which the horn status varies, i.e., in both sexes polled and horned animals occur (e.g., Charolais cattle, Holstein Friesian cattle [12]). There are also sex-linked horns, which are the most common in many sheep breeds (e.g., Romanov sheep [12]).

In cattle and sheep, another form of horn growth is known: scurs. Scurs are hornlike formations that occur occasionally in a wide variety of sizes and forms as an unexpected phenotype when breeding polled cattle or sheep [35,36]. These appendages are smaller, deformed, and not as firmly attached to the skull as normal horns [37]. In goats, the scurs phenomenon has not been proven, but breeders sometimes report similar horn-like structures, such as wiggle horns.

4. Molecular Causes of Inherited Absence of Horns in Domestic Ruminants

The development of horns involves hundreds of genes [2]. Since patterning and differentiation of horn precursor cells occurs early during embryogenesis, it is experimentally difficult or almost impossible to study [38]. Therefore, natural mutations affecting horn growth, such as polledness, offer a valuable alternative for studying the underlying molecular and cellular mechanisms. Numerous studies have shown that the genetic causes of polledness are different in cattle (OMIA 000483-9913), yak (OMIA 000483-30521), sheep (OMIA 000483-9940), and goats (OMIA 000483-9925). The heterogeneity now known suggests that the corresponding mutations affecting different genes occurred independently of each other in the different species. Therefore, the current state of the knowledge on the molecular genetic causes for polledness is described below for each species individually. In particular, it is shown how different the genetic backgrounds are in sheep and goats compared to bovines such as cattle and yak.

4.1. Cattle (Bos taurus and Bos indicus) and Mongolian Yak (Bos mutus)

Polledness in the cattle population has been a known trait for millennia. Hornless dairy cows were already depicted in ancient Egyptian artwork, such as on the sarcophagus of Queen Kawit [39]. The earliest findings of polled cattle in Germany are dated to 4000–6000 years BCE, about 2500 years after the first evidence of domestication [34]. Schafberg and Swalve reviewed that since the 20th century, the occurrence of polled cattle along with the breeding has increased slightly, but is still not in the focus of most developed breeding programs [34]. A recently published review gives a comprehensive overview of the different aspects of inherited polledness in cattle [40]. The *POLLED* locus (*P*) in cattle is located on bovine chromosome 1 [41]. Polledness is inherited as an autosomal monogenic dominant trait, with allele *P* (hornless) dominating allele *p* (horned) [42,43]. Two different *P* alleles can occur in hornless cattle depending on their origin: the "*Celtic*" polled allele (*PC* or P_{202ID}) of Scandinavian and British origin and the "*Friesian*" allele (*PF* or P_{80kbID}), which occurs in cattle of Holstein Friesian origin. Rarely, there are also compound heterozygous animals (e.g., in polled Simmental cattle by crossing with hornless Red Holsteins) [44]. The causal variant of the *PF* allele represents a tandem duplication of an 80-kb segment (Figure 3) [45]. In contrast, the *PC* allele is a smaller-sized complex insertion/deletion variant with a duplication of 208 bp, inserted after 10 bp of the wildtype sequence in combination with a 6-bp deletion [44,46]. Both bovine variants do not affect protein-coding genes but most likely alter the expression of the noncoding RNAs that are relevant for horn bud formation during early embryonal development. Mariasegaram et al. (2010) described the gene networks involved in the development of horns and scurs but did not find differentially expressed genes involved that map to the *P* locus on bovine chromosome 1 [47]. However, a subsequent RT-PCR-based expression analysis revealed the importance of *relaxin/insulin-like family peptide receptor 2* (*RXFP2*) and *forkhead box L2* (*FOXL2*) expression for horn development, as an overexpression was observed in the horn bud area [48]. Interestingly, as detailed later, these two genes are associated with horn growth in sheep and goat, respectively [49,50].

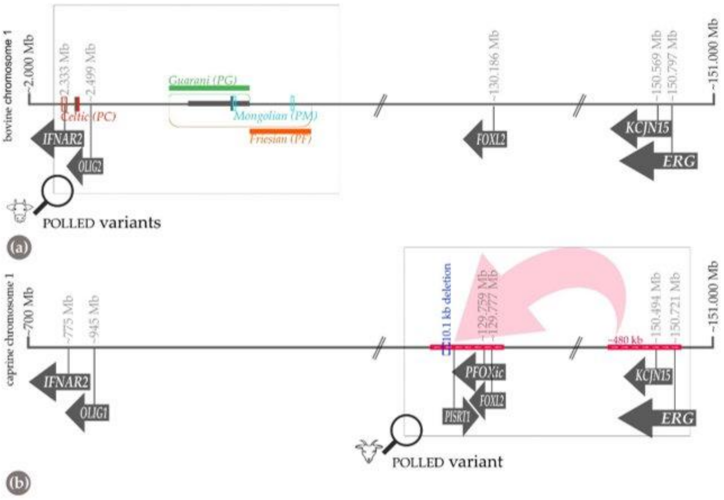

Figure 3. Schematic representation of the location of the different known polledness-causing variants on bovine (**a**) and caprine (**b**) chromosome 1. Note the corresponding genes annotated in both species have been drawn one below the other for simplicity but are in different positions on the respective chromosome. The coordinates refer to the current reference genome of cattle (ARS-UCD 1.2) and goat (ARS1).

In wild yaks (*Bos mutus*), a third "*Mongolian*" allele (*PM* or *P219bpID*) for polledness, affecting the same genomic locus on chromosome 1, was discovered [51]. It is a complex 219-bp duplication/insertion in combination with a 7-bp deletion and 6-bp insertion located 621 bp upstream, resulting in a duplication of an 11-bp motif that is entirely conserved among Bovidae [51] (Figure 3).

The fourth currently known bovine *POLLED* allele, designated as *PG*, was found in polled Nellore cattle (*Bos indicus*). This variant is a 110-kb tandem duplication located in the same genomic region on bovine chromosome 1 (Figure 3) [52].

With the knowledge of the variants explaining the different *POLLED* alleles in cattle, genetic testing is possible, although genotyping of the structural variants, in particular, can be challenging. A detailed comparison of different methods was recently published [53].

Little is known about the genetic background of horns or potential hornlessness in further bovids. For example, in water buffalo (*Bubalus bubalis*), genetically polled individuals are known to occur, but the underlying variant(s) remain unknown (OMIA 000483-89462).

In addition to horned and polled cattle, there are also some with an intermediate phenotype, the so-called scurs or "wiggle horns". Animals showing scurs are heterozygous for one of the polled alleles [48]. The development of scurs in cattle cannot be explained by a single locus, as GWAS studies did not show clear results [36]. The original and still widely accepted model for the inheritance of horns and scurs [42] has recently been rejected. Presumably, an oligogenic model explains the development of scurs in cattle. Capitan (2011) stated that additionally to the type I scurs mentioned by Asai (2004), a quite similar but independent form of scurs (type II) that does not segregate for a known *POLLED* allele was noticed in a single Charolais cattle family. A causative frame-shift variant in the *twist family bHLH transcription factor 1* (*TWIST1*) gene on bovine chromosome 4, representing a loss-of-function allele, was found and highlights the genetic complexity of horn-growth phenotypes in cattle [54].

4.2. Goat (Capra hircus)

As in cattle, polledness in goats (Figure 4) follows a monogenic autosomal dominant mode of inheritance. A complex structural genetic variant characterized by the fusion of a large 480-kb-sized duplicated chromosome 1 segment into the previously reported deleted part of 10 kb further upstream on chromosome 1 [55] causes the absence of horn growth in goats [56] (Figure 3). Recently, the presence of this complex structural variant was also confirmed in Chinese goat breeds with polled animals [57].

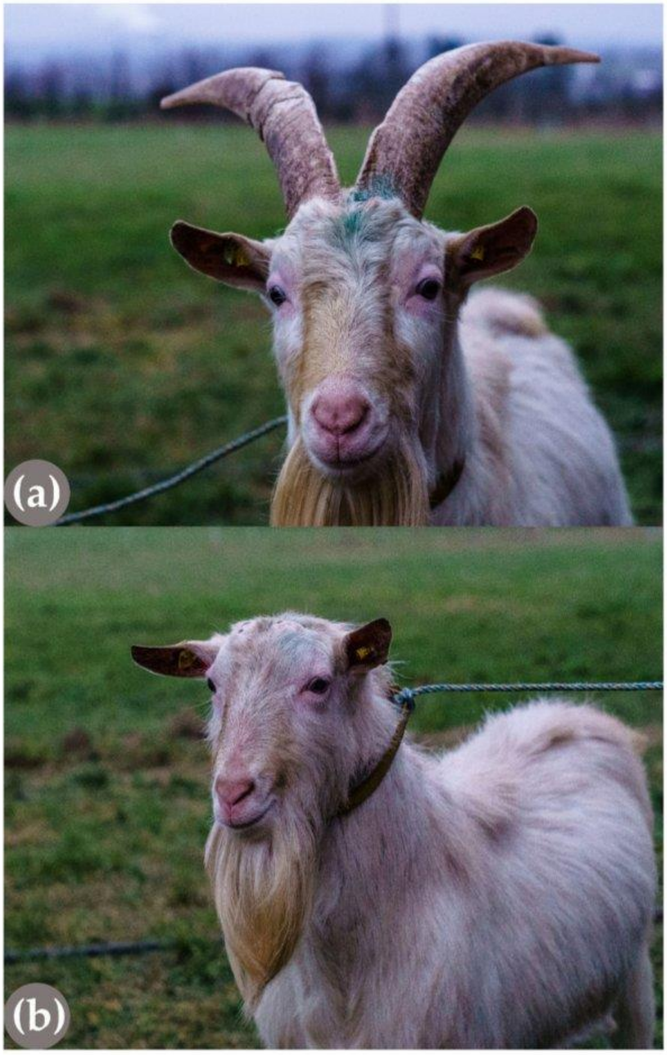

Figure 4. A horned (**a**) and heterozygous polled (**b**) Saanen buck, sired by the same heterozygous polled father. Both rams were about 1.5 years old when the pictures were taken (C. Barth).

So far, it is unclear which elements of these complex structural variants contribute to the lack of horn growth in goats. In contrast to what is known from other species, the dominantly inherited polledness in goats is associated with recessive intersexuality [58] (Table 1). The so-called polled intersexuality syndrome (PIS) was first observed in polled flocks by an abnormal sex ratio, i.e., a higher-than-average number of phenotypic males [59,60]. Homozygous polled females (60, XX) are infertile intersexes. They show a variable phenotype ranging from "normal" female to "normal" male, including all possible combinations in between (Figure 5). Therefore, it is difficult to identify some of these hornless intersexual animals as such [61]. Generally, intersexuality is not observed in genetically male (XY) homozygous polled goats. However, there is non-scientific evidence that hornlessness can also be associated with fertility problems in bucks. This is currently being investigated. Due to the known inheritance, it is possible to the avoid excessive appearance of PIS-affected offspring by well-planned breeding in goats (e.g., [62]), but the establishment of a fully polled and fertile flock is still impossible. Due to these new findings on the molecular background of polledness in goats, genetic testing for PIS is now possible as well. With genotyped animals, planned breeding is facilitated and polled intersexes with an inconspicuous phenotype of the genitalia can already be identified early in life [56,57].

Table 1. Impact of the caprine *POLLED* allele on horn status and the fertility of female (XX) and male (XY) goats.

	Genotype at the *POLLED* Locus		
Genetic Sex	**pp (Homozygous; Wild Type)**	**Pp (Heterozygous)**	**PP (Homozygous)**
XX—female	horned/fertile	polled/fertile	polled/infertile—intersex (normal outer phenotype or "pseudo-buck" to variable degrees
XY—male	horned/fertile	polled/fertile	polled/fertility unclear

Figure 5. Comparison of the external genitalia of a normal (**a**) and PIS-affected (**b**) female (XX) homozygous polled Saanen goat.

Functionally, the originally reported PIS-causing 11-kb deletion published by Pailhoux et al. (2001) affects elements that regulate the transcription of *FOXL2*, which is also implemented in the polledness of cattle as mentioned above [55,63]. Obviously, homozygosity for the deletion leads to a decreased transcription of these genes in the goat's ovaries [55]. The recently described refined breakpoints in that region of goat chromosome 1 are located in the *FOXL2* topologically associating domain (TAD) when compared to the corresponding human genome region, i.e., in the regulatory domain responsible for *FOXL2*. Duplications of genomic regions are associated with various disorders, but the phenotypes, which are thought to arise from an increase in gene copy number, often cannot be explained by changes in gene dosage [64]. However, genomic duplications that change the structure and function of topologically associated domains (TADs) can cause phenotypes without altering the gene copy number [65]. TADs are chromosomal regions with an increased frequency of internal chromatin interactions, e.g., between genes and their distal regulatory elements.

In humans, the duplication of a region between *SRY-box transcription factor 9* (*SOX9*) and *potassium inwardly rectifying channel subfamily J member 2* (*KCNJ2*) that lies within the *SOX9* TAD results in sex reversal from female to male. In contrast, an inter-TAD duplication that involves sex reversal duplication and spans into the *KCNJ* TAD—but without the *KCNJ* genes included—has no influence on the phenotype [65]. In goats, the duplicated 480 kb-sized genomic segment of the PIS-associated variant contains the *KCNJ* gene and parts of the *ETS transcription factor ERG* (*ERG*) gene as well as parts of the respective TADs. Therefore, the duplicated segment contains a boundary that separates the TADs and, thus, the regulatory domains from each other. When the duplicated segment is inserted into the breakpoint of the *FOXL2* region, it can be assumed that a fusion TAD (neo-TAD) is formed, consisting of one part of the duplication and the remaining of the *FOXL2* TAD. Due to the inversion, *KCNJ* is placed on the other side of the boundary and is, therefore, isolated (Figure 3). Therefore, it could be speculated that the "residual" of the *ERG* gene is of functional importance. This part also contains enhancers and could, therefore, lead to ectopic expression of *FOXL2* in developing horn buds. Future research might evaluate the hypothesis that the caprine PIS variant represents a loss-of-function of *FOXL2*, as parts of the regulatory domain are missing, leading to ectopic expression in addition to the presence of a gain-of-function through the *ERG* enhancers.

4.3. Sheep (Ovis aries)

Polledness in sheep is an interesting trait not only from a breeding but also from an evolutionary point of view. In most contemporary production sheep breeds, almost all animals are polled (absence of horns), while horns are found mainly in autochthonous breeds. Some sheep breeds, such as the Poll Dorset were specifically bred after the model of the horned basic breed (Dorset Horn), only hornless. Besides sheep breeds fixed either for horns or polledness, there are also those in which one or both sexes have a variable horn status, and even those in which rams are always horned and females are always polled (Table 2, Figure 6). Considering the representatives of the first breed panel of the International Sheep Genome Consortium (ISGC) as a cross-section of the total population (including wild sheep), it can be seen that the majority of the breeds (~39%) are completely hornless, in ~28% of the breeds, the horn status is fixed in one sex and variable in the other, completely variable horn status is present in ~13% of these breeds, whereby strictly sex-specific horns account for 12%, and in only 8% of the breeds studied, all individuals are horned [12,66]. Therefore, the inheritance of horns in sheep varies according to breed and is more complicated than in goat and cattle. Initially, a model with three alleles was proposed, as horn growth in sheep was thought to be controlled by a single autosomal locus [37,67]. The mode of inheritance differs between sexes and it was proposed that the allele that results in horns is dominant in males and recessive in females [68].

Recent results in Merino sheep confirmed the influence of sex on horn status in this breed [69]. Independent genomic analyses pointed towards a single autosomal locus on chromosome 10 harboring the variant that causes polledness in sheep [68,70]. Pickering et al. (2010) identified a 1.8-kb insertion in the 3′-untranslated region of the ovine *RXFP2* gene located in this region (Figure 7), which was also independently described by Wiedemar and Drögemüller (2015) and present in polled sheep only [49,71]. For Merino sheep, two highly significant associated SNP markers (OAR10_29546872.1, OAR10_29458450) were found near the 1.8-kb insertion, but they still cannot fully explain the genetic diversity regarding the presence/absence of horns in this breed. However, Duijvesteijn et al. (2018) stated that if genotype GG at the marker OAR10_29458450 or TT at marker OAR10_29546872.1 is taken into account, a reliable prediction of non-horned male Merino sheep is possible.

Table 2. List of sheep breeds (*Ovis aries*), including the European mouflon (*Ovis musimon*), showing different horn phenotypes between sexes. Individuals of the respective sex and breed are either horned, polled, or the horn status is variable (there are polled, horned, and scurred individuals).

Horn Status Group	Breed	Species	Horn Status Females	Horn Status Males
Completely polled	Barbados Blackbelly Sheep *	*Ovis aries*	Polled	Polled
	Bentheimer	*Ovis aries*	Polled	Polled
	Charollais	*Ovis aries*	Polled	Polled
	Coburger	*Ovis aries*	Polled	Polled
	East Friesian Milk Sheep *	*Ovis aries*	Polled	Polled
	German Black-headed Mutton	*Ovis aries*	Polled	Polled
	German Brown Mountain	*Ovis aries*	Polled	Polled
	German White Mountain	*Ovis aries*	Polled	Polled
	Ile de France	*Ovis aries*	Polled	Polled
	Kerry Hill Sheep *	*Ovis aries*	Polled	Polled
	Lacaune Sheep *	*Ovis aries*	Polled	Polled
	Merinoland Sheep *	*Ovis aries*	Polled	Polled
	Poll Dorset	*Ovis aries*	Polled	Polled
	Roughwool Pomeranian Sheep *	*Ovis aries*	Polled	Polled
	Rhone Sheep	*Ovis aries*	Polled	Polled
	Suffolk	*Ovis aries*	Polled	Polled
	Texel Sheep *	*Ovis aries*	Polled	Polled
Completely horned	Grey Horned Heath *	*Ovis aries*	Horned	Horned
	Scottish Blackface Sheep *	*Ovis aries*	Horned	Horned
	Valais Blacknose Sheep	*Ovis aries*	Horned	Horned
	Mouflon *	*Ovis musimon*	Horned	Horned
Variable in both sexes	African Dorper Sheep *	*Ovis aries*	Variable	Variable
	Alpines Steinschaf	*Ovis aries*	Variable	Variable
	Icelandic Sheep	*Ovis aries*	Variable	Variable
	Krainer Steinschaf *	*Ovis aries*	Variable	Variable
	Soay Sheep *	*Ovis aries*	Variable	Variable
Strictly sex-linked	Ethiopian Menz	*Ovis aries*	Polled	Horned
	Cameroon Sheep *	*Ovis aries*	Polled	Horned
	Rambouillet	*Ovis aries*	Polled	Horned
Males horned, females variable	Walachian Sheep	*Ovis aries*	Variable	Horned
	Ouessant Sheep *	*Ovis aries*	Mostly polled	Horned

* Note that individuals of the marked breeds were genotyped as wild type for the goat PIS-related complex variant.

However, it was recognized that this polledness-associated 1.8-kb insertion variant, which adds a potential antisense RNA sequence of *eukaryotic translation elongation factor 1 alpha 1* (*EEF1A1*) to the 3′-end of *RXFP2* transcripts, does not segregate perfectly with the polled phenotype in sheep breeds with variable or sex-linked horn status [72]. Therefore, it was concluded that the observed variant cannot be the only cause of polledness in sheep. Nevertheless, as far as we know, no other polled-associated alleles have been discovered in sheep so far (OMIA 000483-9940).

The rams of the African Dorper sheep breed can have normal horns or scurs or be hornless, whereas female Dorper ewes are scurred or polled. Interestingly, this breed is fixed for the *RXFP2*-related 1.8-kb insertion [72]. Publicly available short-read whole-genome sequencing data from nine male Dorper sheep with known different horn statuses (four horned, three scurred, and two polled, Table S1) were explored using the current sheep reference genome assembly (ARS-UI_Ramb_v2.0) to search for possible additional *RXFP2*-associated alleles. A visual inspection of the region of the *RXFP2* gene using the integrated genome viewer (IGV) [73] revealed no evidence of novel variants (data not shown). Nevertheless, it might be helpful to use other techniques, such as long-read sequencing, to study the region of interest with a focus on more complex and structural variants, as this was also the successful approach to uncovering the genetic features of the complex PIS-associated variant in goats [56].

Figure 6. Sheep breeds (*Ovis aries*) showing different horn phenotypes belonging to different horn groups. Rhone Sheep (**a**) and Merinoland Sheep (**b**) (C. Barth) are typical representatives of the group of completely polled breeds. On the contrary, in breeds in which horns are fixed, both individuals develop horns, exemplified by a ewe (**c**) and a ram (**d**) of the German Grey Heath sheep breed. As an example of a breed in which horn status is variable in both sexes, two Icelandic sheep rams, horned (**e**) and hornless (**f**), are shown (K. Elísabetardóttir). In other breeds, the horn status is linked to the sex. For example, in the Cameroon sheep (**g**), the rams are always horned (ram on the left side and male lamb in the middle) and the females are always hornless (ewe in the middle and on the left side of the picture). Please note that a list of sheep breeds belonging to different horn status groups can be found in Table 2.

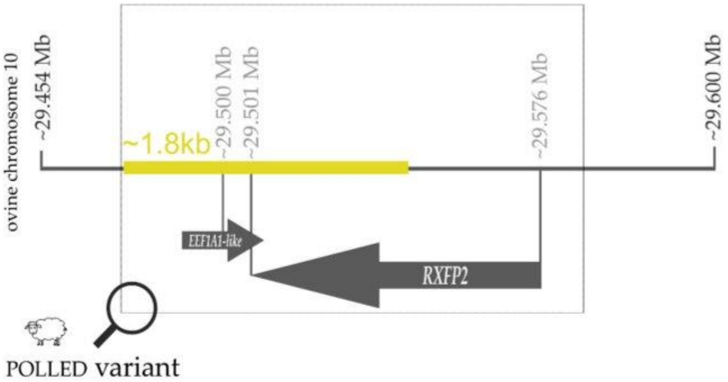

Figure 7. Schematic representation of the polledness-associated insertion variant in sheep in the 3′UTR region of the RXFP2 gene on chromosome 10. The coordinates refer to the current reference genome ARS-UI_Ramb_v2.0 of the sheep. Note that this variant is not associated with polledness in breeds with a sex-linked or variable horn status.

The localization of the homologous sequences of the caprine PIS region ruled out the possibility that intersexuality in sheep has the same or similar underlying genetic causes as in goats [74]. However, there is also no evidence of a comparable relationship between polledness and intersexuality in sheep. A small test series (14 breeds, 26 individuals, Table S2) in our laboratory has shown that the application of the published PCR-based detection of the caprine PIS-associated variant using genomic DNA from sheep revealed only the presence of the wild type allele (data not published), supporting the results of Li et al. (2020).

In addition to the mere presence or absence of horns (including scurs), the expression of horn shape and size in sheep also varies. In studies of the isolated Soay sheep population on St. Kilda, the locus for these continuous traits could also be located in the *RXFP2* gene region on chromosome 10 [31]. This suggests that other or even all variants affecting horn growth in sheep may be related to the gene region around *RXFP2* [15,31]. Whole-genome sequencing of Chinese sheep breeds found eight *RXFP2*-related markers that segregate, at least partly, with horn morphology, in terms of length and shape [32].

5. Recent Developments in Genetic Engineering Offer New Possibilities for Breeding Hornless Ruminants—First Examples and Current Legal Limits

Even though European law strictly limits the use of new genetic engineering since the landmark ruling of July 2018 [75], the so-called "genetic scissors" techniques, such as transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR [76]) are still seen as a great opportunity in agricultural science. The principle behind the application of these nucleases is to trigger a DNA double-strand break at a previously defined location in the genome, which is repaired in the cells in one of two possible ways. Firstly, there is the repair mechanism of non-homologous end-joining in which resulting fragments are ligated without an external template, enabling gene knock-outs. Secondly, there is the homology-directed repair, in which a predesigned template is used as a pattern for ligating the fragments. This makes this method suitable for gene knock-in or the replacement of a specific sequence in general [77–79].

As one of the first successful applications in domestic animals, the "*Celtic*" polled allele (*PC*) of cattle was independently integrated into the genomes of horned cattle [80–82]. Moreover, it was shown that all heterozygous progeny of dairy bulls that became homozygous for *PC* after genome editing expressed the polled trait as expected. At the same time, other intended changes in the genome sequence were not detectable [83].

Since polledness in cattle is apparently not associated with any harm, it is possible to spread the desired trait through conventional breeding within a few generations in previously horned populations without any negative effects. However, to give genome editing an edge, some argue that the population of hornless breeding bulls with reliable and good breeding values (comparable to horned counterparts) is too small to avoid inbreeding while supporting the introgression of the polled trait. For this reason, it takes much longer to increase the frequency of polledness in a population using conventional breeding strategies than with genetic engineering [84–86]. From a basic science perspective, since it appears that more than one variant affecting different genes is causing polledness, genome editing might give the opportunity of demonstrating which of the variants is crucial for the absence of horns. Alternatively, confirmation of the interaction of several variants as a cause for polledness would be possible through it, as recently done for the PC variant in cattle [87].

These techniques have also been successfully applied to sheep and goats when dealing with issues other than polledness [88,89]. For these two species in particular, and the unique features of the inheritance of polledness in them, the use of genome editing would offer new approaches and opportunities to establish the desired trait in the respective population. In goats in particular, it might be helpful to find a way to introduce inherited polledness into the population without the associated intersexuality. One possibility would be an attempt to insert one of the bovine variants for polledness, preferably the less complex *PC* variant, into the goat genome. So far, no such attempts have been published.

However, in addition to strict legislation in Europe, there are also reservations among the public about genetically modified animals for consumption. Both ethical concerns and risk–benefit assessments of genetic engineering in food production explain the critical attitude of consumers. In general, it can be summarized that Europeans are more critical of the topic than American and Asian consumers [90–92]. Finally, there are also critical voices that claim, on the basis of these supposed examples of success with hornless cattle, that this is the wrong way to solve the problem, as it is merely a "technological solution" to a complex social problem [93].

6. Conclusions

Although the presence or absence of horns is a trait as old as livestock, it is a topic that never loses its relevance. The study of horn phenotypes in ruminants confirms the still underestimated role of domestic animals as unique models for biomedical research due to their long history (thousands of years) of strong phenotypic selection. The three known hornless loci in cattle, goat, and sheep each affect different genes, although the resulting phenotypes with the absence of horn growth are more or less identical. This confirms the assumed heterogeneity and complexity that determines the development of these organs, which are unique in the animal kingdom. Nevertheless, the underlying genetic mechanisms, especially in sheep, remain largely unknown, highlighting the need for further research in this field. A challenge will be to clarify the implementation of intermediate phenotypes, such as scurs and sex-linked factors. In addition, the underlying mechanisms in all three ruminant species still need to be investigated. There is no doubt that breeding for polledness is a sensible and permanent alternative to surgical dehorning in order to take animal welfare into account and offer an animal-friendly alternative [27,84].

The new possibilities offered by genome editing techniques could serve as a tool to spread this trait faster than through conventional breeding, especially in cattle. In goats, it may be possible to specifically modify the corresponding genomic regions that are altered in cattle or sheep to avoid the negative association of the naturally occurring hornless variant with intersexuality.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/genes13050832/s1, Table S1: Whole-genome sequencing data from Dorper sheep.; Table S2: Sheep genotyped with genetic testing for polled intersex syndrome in goats.

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3.3. Third publication

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Original study

Analysis of genetic variants for different horn phenotypes and their inheritance in Icelandic sheep

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Abstract. Icelandic sheep are characterized by a great diversity in horn phenotypes. Within their breed, they show a variability in terms of this trait to an extent rarely observed elsewhere. Previously, several genetic variants were published as markers for horn status (in terms of absence or presence of horns, including scurs) and horn traits (e.g., oval horns, horn length and polyceraty). The aim of this study was to genotype, for the first time, five of these genetic variants in Icelandic sheep with different horn phenotypes, as well as to analyze their inheritance. Phenotypic and pedigree data, as well as DNA samples from two Icelandic sheep farms, were used. Genetic variants were genotyped by published PCR-based methods in all samples ($n = 94$) or in subsets. As in other sheep breeds with variable horn status, the inheritance of the presence or absence of horns was shown to be complex in Icelandic sheep, especially when sheep carry anything other than regularly formed horns. The 1.78 kb sized *RXFP2* insertion on ovine chromosome 10 previously described to be associated with polledness in several sheep breeds was also found to be present in Icelandic sheep and showed some association but not a perfect segregation with the individuals' horn statuses. Missing associations were especially seen in sheep with scurs and oval horns. Regarding horn shape, there was no agreement with the studied variants described in Chinese breeds having comparable horn traits. However, matching tendencies were seen for the horn size variant that was found in the same study. All sheep with four or more horns carried the already published 4 bp deletion in *HOXD1*, as previously described for three other sheep breeds. Interestingly, for the first time, the deletion was also detected in phenotypically polled animals originating from multi-horned families. According to the results from animals genotyped simultaneously for the *RXFP2* and the *HOXD1* variants, polledness in sheep with a genetic disposition for polyceraty seems not to be controlled by the *RXFP2* insertion. However, this and all other findings in Icelandic sheep need to be confirmed by analyzing a higher number of well-phenotyped animals.

1 Introduction

Iceland, due to its isolated island location and strict import restrictions for animals, is a particularly interesting area for research. One example of an interesting research object is the northern European short-tailed Icelandic sheep (short: Icelandic sheep), which were originally formed by various northern European breeds brought to the island by the Viking settlers between 800 and 1000 AC (Dýrmundsson and Niżnikowski, 2010). It is the only existing sheep breed in Iceland today and has not been crossed with foreign breeds for centuries (Eythorsdottir et al., 2008; Dýrmundsson and Niżnikowski, 2010). A recent diversity study shows that the genetic influence of foreign breeds imported only occasionally in the past is negligible for the recent Icelandic sheep (data not shown, publication in preparation). To some extent, this is comparable to the much-studied population of feral Soay sheep in the archipelago of St. Kilda, Scotland (Clutton-Brock and Pemberton, 2009). Nevertheless, the Icelandic sheep show a great phenotypic variability with respect to different traits (Porter et al., 2016). A striking characteristic is the horn phenotype, which seems to be polymorphic in males and females (Fig. 1).

In the inheritance of horns or polledness, the *RXFP2* gene on ovine chromosome 10 plays a major role (Wiedemar and

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Drögemüller, 2015; Pickering et al., 2009), although it has already been shown for some breeds with variable or sex-linked horn status that the published 1.8 kb insertion in the 3'-UTR region of this gene is not associated with polledness (Lühken et al., 2016; He et al., 2016). Duijvesteijn et al. (2018) succeeded in the genomic prediction of the presence or absence of horns in Merino sheep using two highly significant single nucleotide variants (SNVs) on ovine chromosome 10 (OAR10_29458450 and OAR10_29546872.1) as markers. Evidence of one of the two is already considered to be sufficient for the prediction, but this has only been proven in Merino sheep (Duijvesteijn et al., 2018). A total of 68 genes were identified recently that show a down- ($n = 10$) or up-regulation ($n = 58$) during horn bud development in sheep embryonic development (Luan et al., 2023). Luan et al. (2023) state that the results of the expression analyses indicate that only a few genes are involved in horn development – including the often-mentioned *RXFP2*.

In addition to polled ("kollótt", Fig. 1a; scured, Fig. 1b–c), and horned ("hyrnt", Fig. 1d–e) individuals, there are also Icelandic sheep that carry a multitude of horns (four to six horns, polyceraty) (Dýrmundsson, 2005). Interestingly, those can also be polled or scured. Breeders are able to differentiate between polled sheep of two-horned origin and of polycerate origin based on the shape of the skull.

The cause for the evolution and persistence of the polyceraty trait has not yet been explained. It is assumed that the emergence of supernumerary horns is the result of a split in the horn buds during embryo development (Allais-Bonnet et al., 2021). The dominant trait of polyceraty in sheep was recently shown to be associated with a short deletion (4 bp sized) in the *HOXD1* gene (Allais-Bonnet et al., 2021) after it had been mapped on ovine chromosome 2 previously, which was confirmed by GWAS for Damara, Jacob, and Navajo-Churro sheep (Kijas et al., 2016; Greyvenstein et al., 2016). The association with a region on chromosome 2 was confirmed for three Chinese breeds as well (He et al., 2016; Ren et al., 2016).

In addition to the presence or absence and/or the number, the shape and size of the horns can vary in Icelandic sheep as well. One can find oval horns that do not have sharp edges in cross-section but also normal "spiral" ones in both sexes (Fig. 1d–f). The same region in which the *RXFP2* gene is located was found to be associated with the horn type and base circumference in male Soay sheep (Johnston et al., 2010). In the same region, a quantitative trait locus (QTL) for the dimension of horns has been found in bighorn sheep, *Ovis canadensis* (Kardos et al., 2015; Poissant et al., 2012). A haplotype within and around the *RXFP2* gene, specifically one SNV (OAR10_29461968) of this haplotype, was shown to segregate with horn length, as well as with horn shape, in an investigation with different Chinese sheep breeds (Pan et al., 2018).

The aim of this study was to analyze, for the first time, the previously mentioned genetic variants known or suspected to influence different horn phenotypes (Table 1), as well as the inheritance of horn phenotypes in Icelandic sheep.

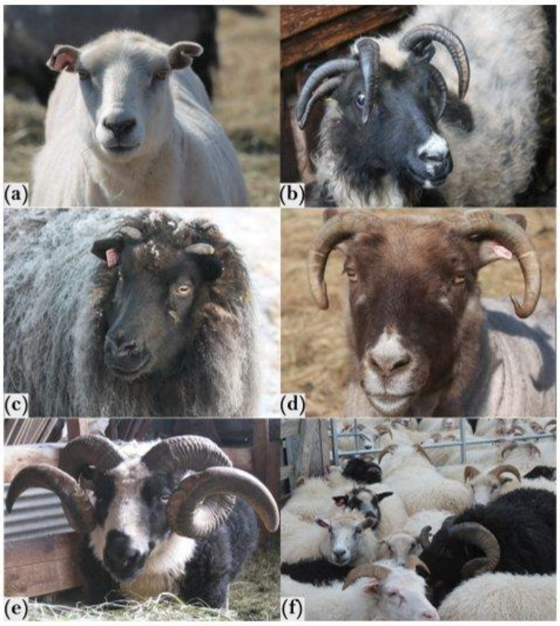

Figure 1. An example for the diversity of the horn status in Icelandic sheep of both sexes. **(a)** Polled mutton. **(b)** Polycerated ewe (six horns). **(c)** Ewe with scurs (horn-like structures). **(d)** and **(e)** Different horn shapes in rams: oval **(d)** and normal "spiral" horns **(e)**. **(f)** A flock with polled and horned individuals.

2 Material and methods

2.1 Animals

In total, samples from 94 Icelandic sheep were collected. Samples from 61 sheep (26 males and 35 females) originated from a single farm in Iceland (no. 1). Furthermore, pedigree information from nine additional sheep was used, but no DNA samples were available. As, in that specific farm, no polycerate sheep were available, we additionally received samples from 33 sheep from another Icelandic farm with a known presence of polycerate sheep (no. 2). This sample set contained both multi-horned (four to six horns) and normally horned sheep, as well as polled ones belonging to those two groups. Further detailed information on the animals used for the analyses can be found in Table 2.

Sample collection was initially performed for diagnostic purposes (scrapie eradication program), and remainders were provided to us for further use.

Table 1. Overview of previously published variants used for genotyping of horn-related phenotypes in Icelandic sheep.

Name position	Gene	Ovine chromosome	Associated with	Breed(s)	Reference
1.78 kb insertion g. 29 456 048–29 457 880 (OARv3.1)	*RXFP2*	10	Polledness	Bündner Oberländer sheep, Valais Red sheep, Valais Blacknose sheep, Engadine Red sheep, Swiss Black-Brown Mountain sheep, Swiss Mirror sheep, Swiss White Alpine sheep	Wiedemar and Drögemüller (2015)
OAR10_29458450 (TT)	Close to *RXFP2*	10	Polledness	Merino sheep	Duijvesteijn et al. (2018)
OAR10_29461968 (TT)	*RXFP2*	10	Increased horn length	Oula sheep, Prairie Tibetan sheep, Valley Tibetan sheep, Small Tail Han sheep	Pan et al. (2018)
OAR10_29461968-OAR10_29462010 (TT, "haplotype 2")	*RXFP2*	10	Horn shape → curled rather than oval	Oula sheep, Prairie Tibetan sheep, Valley Tibetan sheep, Small Tail Han sheep	Pan et al. (2018)
4 bp deletion (AGTA/–) g.132,832,249-132,832,252del (Oar_v4.0 assembly)	*HOXD1*	2	Polyceraty	Jacob sheep, Navajo-Churro sheep, Damara sheep	Allais-Bonnet et al. (2021)

Table 2. Overview of sheep samples used according to general and, where necessary, detailed horn status, as well as sex.

Farm	General horn status	*n*	Sex	*n*	Details on horns	Sex	*n*
No. 1	Polled (kollótt)	31	Female Male	26 6			
	Horned (hyrnt)	21	Female Male	3 18	Oval (sívalhyrnt)	Female Male	1 11
					Normal (hyrnt)	Female Male	2 7
	Scurs (smáhnýflótt)	9	Female Male	6 3			
No. 2	Polyceraty	33	Female Male	13 20	Four to six horns	Female Male	2 2
			Unknown	1	Polled (four to six horns)	Female Male Unknown	2 1 1
					Two horns	Female Male	5 14
					Polled (two horns)	Female Male	4 2

2.2 DNA extraction

Depending on the sample type, DNA was extracted with either a blood kit or a tissue kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. Only the amount of used elution buffer for blood samples was lowered to 75 µL in order to yield a higher DNA concentration.

2.3 Pedigree

The pedigrees for farm no. 1 were created according to the owner's information about the relatedness of the animals, supported by the herd book information. Complete pedigree information, including information about the parents' horn status, up to the third or fourth generation, was available for most of the sheep from this farm (no. 1); it was only for four male and five female sheep of the first and second generations that no horn status information was available. To demonstrate the inheritance of horn status in Icelandic sheep, focusing on polled matings and scured offspring, including the influence of the previously published *RXFP2* variant (Wiedemar und Drögemüller, 2015), two partial pedigrees were constructed with the help of QuickPed (Vigeland, 2022). For the second farm, no information on the parents was available; therefore, no pedigree was drawn.

2.4 Genotyping

A total of 94 out of 94 samples were genotyped for the *RXFP2* variant (1.78 kb insertion). Genotyping of the three additional variants (details can be found in Table 1) was performed for a selection of the samples. It was ensured that all horn phenotypes were represented, but the focus was on individual phenotype groups: for genotyping of the haplotype published by Pan et al. (2018), the focus was on the horned individuals, with records of their horn form, including some polycerate ones. In total, 40 individuals were genotyped for these variants. For the polledness predicting SNV in merino sheep (Duijvesteijn et al., 2018), 55 individuals were genotyped, with the focus being on polled versus horned sheep (regardless of further horn characteristics). The *HOXD1* variant published by Allais-Bonnet et al. (2021) was mainly genotyped in the sheep from the polycerate flock; however, in addition, some polled and horned sheep were analyzed for comparison. This resulted in a total of 20 individuals.

For genotyping, PCR protocols as published elsewhere (Lühken et al., 2016; Pan et al., 2018; Duijvesteijn et al., 2018; Allais-Bonnet et al., 2021) were used with slight modifications and can be found in Table S1 in the Supplement.

3 Results

Seen as a whole, the pedigree information did not resolve the question of the inheritance mode of horn status. We found that the presence or absence of horns or scurs across the pedigree did not follow that of a simple monogenic trait. Most consistent is the very frequent occurrence of polledness among offspring from polled × polled matings (12 out of 15, Table S2). However, there are exceptions from that pattern. For example, among the six matings of polled parents displayed, four resulted in polled offspring, whereas two resulted in two male offspring with oval horns and scurs (Fig. 2) and a single male with scurs (Fig. 3).

Matings involving at least one oval-horned parent resulted in a polled female (Fig. 2), a normally horned male (Fig. 2), or even a scured male (Fig. 3) offspring. Also, a polled offspring of oval-horned parents was not observed in the sample set. A mating of two scured parents did not take place in the analyzed group of sheep. Table S2 gives a complete overview of the horn phenotype of offspring resulting from matings of parents with different combinations of their horn phenotype.

The 1.78 kb sized *RXFP2* insertion (ins) shown previously to be associated with polledness (Wiedemar and Drögemüller, 2015) was found to be present in Icelandic sheep and showed some association but not a perfect segregation with the individuals' horn statuses (Table 3). In all cases where genotyping was possible, the genotype of the offspring matches the expectation based on the genotype of the parents (Figs. 2 and 3). Except for a single polled sheep of the polycerate family, a consistent pattern is the presence of the insertion at least on one chromosome in all polled and scured sheep. In line with this, the majority of normally horned sheep (13 out of 16 males, 5 out of 6 females) did not carry the insertion at all. However, some normally horned sheep were heterozygous or homozygous for the insertion.

In contrast to normal horns, oval horns were not observed in sheep without the *RXFP2* insertion.

In sheep from polycerate families, the *RXFP2* insertion was not found to be present in the homozygous state. For four animals, the genotyping failed even after repetition. However, based on the pedigree information, it was possible to deduce the most likely *RXFP2* genotype for three animals (indicated by * in Figs. 2 and 3).

All 55 animals genotyped for the polledness predicting SNV in Merino sheep showed the wild-type allele (A); thus, there was no segregation of this variant with the examined horn status.

In the analyzed sheep, the SNV OAR10_29462010 appears to be fixed as only allele C is present. SNV OAR10_29461968 was found to be variable: C homozygotes were only found in two males with oval horns and a single female polled sheep. T homozygotes were found in all but the polled individuals tested and seem to be most frequent in normally horned animals. (Table S3).

Haplotype 2 (TT), found in Chinese breeds with curled (normal) horns, was not present in the analyzed samples, regardless of the horn phenotype. Hence, no segregation of the previously published haplotypes with a certain horn form was found.

Table 3. Distribution of the occurrence of the 1.78 kb sized insertion in *RXFP2* depending on horn phenotype and sex of the analyzed Icelandic sheep. Please note that sheep from farm no. 2 that had no documentation of multi-hornedness were added to the respective horn phenotype group (polled or normally horned).

Horn phenotype	Sex	*RXFP2* genotype (1.78 kb insertion)		
		–/–	ins/–	ins/ins
Polled (kollótt)*	Female		9	18
	Male		1	7
Normally horned (hyrnt)	Female	5	1	
	Male	13	2	3
Scurs (smáhýflótt)	Female		6	
	Male			3
Oval horned (sívalhyrnt)	Female		1	
	Male		8	3
Polycerate (four to six horns)*	Female	1		
	Male		1	
Polled polycerate (four to six horns)	Female	1	1	
	Male		1	
	N/A		1	

* *RXFP2* genotyping failed for two additional polled (females) and polycerate sheep (one of each sex). N/A – not analyzed.

All polycerate sheep (four- and six-horned) and five polled sheep from polycerate families were carriers of the 4 bp deletion in *HOXD1* in either a heterozygous or a homozygous state. Neither two-horned nor polled individuals from non-polycerate families (farm no. 2) carried this variant (Table 4). The same applies to the genotyped animals from farm no. 1: none carried the *HOXD1* variant.

4 Discussion

Concerning Soay sheep, polledness is recessive, and males that are heterozygous in terms of the horns locus are horned, while heterozygous females carry scurs (Johnston et al., 2009). Also in the investigated families of Icelandic sheep, mating of polled parents mostly led to polled offspring, but there were few exceptions from this sign of a recessive trait. Moreover, in contrast to what had been observed in Soay sheep, scurs were not only limited to female Icelandic sheep, and this was also not a common outcome of horned × polled matings. Instead, scured males where observed in the sample set and were derived from each parental phenotype combination: both parents polled, both horned, or a horned father. These observations contradict parts of the most recent report (Johnston et al., 2009) about the mode of inheritance of polledness in sheep. Of course, for statistical approval or disapproval of any inheritance pattern, the sample set is too small, and, in some cases, the phenotypic data (horn status) of the parents were not documented. As it is possible that the mode of inheritance varies between breeds, the mode of inheritance in Icelandic sheep should be determined in a larger sample set in follow-up investigations.

As the Icelandic sheep is a breed with a variable horn status, it was expected that the 1.78 kb *RXFP2* insertion (Wiedemar and Drögemüller, 2015) would not segregate perfectly with polledness. However, in contrast to Dorper and Bovec sheep, which show a variable horn status but seem to be fixed with regard to the *RXFP2* insertion (Lühken et al., 2016), all three possible genotypes were observed in the Icelandic sheep. Most of the polled Icelandic sheep are homozygous with regard to the insertion, whereas the vast majority of horned individuals are homozygous with regard to the wild type, thus fitting more or less to what was observed for the *RXFP2* variation in uniformly horned or polled breeds (Lühken et al., 2016; Wiedemar and Drögemüller, 2015; Pickering et al., 2009). Yet there were exceptions from that rule. Heterozygous sheep do not fit the scheme at all as this genotype was found in male and female polled, horned, and scured (except males) sheep. In addition, sheep with oval horns do not fit into the scheme as there was no oval-horned individual without the *RXFP2* insertion. Maybe this horn phenotype is independent from the *RXFP2* variant or, in contrast, is only expressed in individuals with at least one copy of the *RXFP2* insertion. However, to prove this, a larger sample set would be needed. In comparison to the other horn phenotypes, the inheritance of both oval horns and scurs is the least comprehensible. Taken together, an influence of the *RXFP2* insertion on horn status (in terms of the presence or

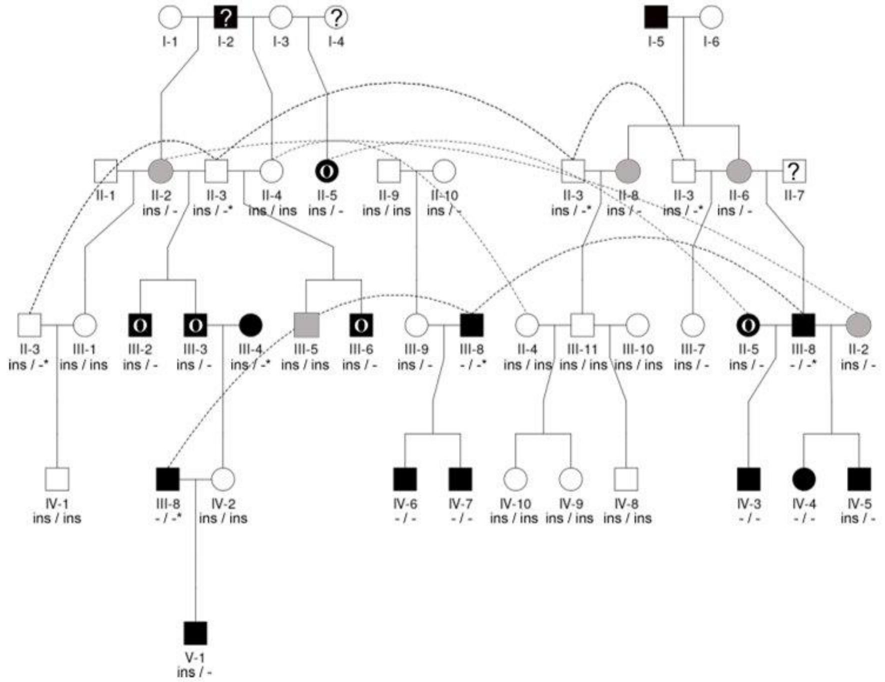

Figure 2. The pedigree displays the inheritance of horn status in Icelandic sheep genotyped for the *RXFP2* insertion, with the focus being on matings of polled parents. The pedigree shows five generations (I-V) including available horn status information (gray: scurs, white: polled, black: normally horned, black with O: oval horned, black with ?: horn form unknown, ?: unknown), *RXFP2* genotype (* indicates suggested genotype), and sex (circle: female, square: male). Dashed lines indicate the same animal in different matings, e.g., the breeding ram II-3. Please note that polled matings in the Icelandic population not only result in polled offspring (IV-1, III-9, IV-8-10) but also result in oval-horned (III-6) and scured progeny (III-5).

absence of horns or scurs) cannot be ruled out, but this is not seen consistently in Icelandic sheep. Among the breeds analyzed by Lühken et al. (2016), the Bavarian Forest breed showed the greatest similarity with the Icelandic sheep analyzed here in terms of variability of horn status and the *RXFP2* variant. Based on this, it is also not surprising that the SNV OAR10_29458450 close to the above-mentioned insertion, which can be used as a polled-predicting variant in Merino sheep (Duijvesteijn et al., 2018), seems not to be a suitable marker for horn status in Icelandic sheep. Only the wild type was found in the investigated sheep, regardless of their horn status. Taken together, the current findings can be considered to be an indication that more than just one gene locus influences the horn status in sheep, as has also been seen in cattle (scurs: Gehrke et al., 2020; Tetens et al., 2015, polledness: Nicholas and Tammen, 2023a, reviewed by Simon et al., 2022). Based on the evolutionary history of the Icelandic sheep breed, it is very likely that they could carry several different variants influencing horn traits.

As information about the horn morphology of the sampled horned sheep was available, we examined a possible association with the previously published haplotype that showed a segregation with horn size and form in Chinese breeds (Pan et al., 2018). These also showed either rather spiral or oval horns, comparable to horn shapes occurring in Icelandic sheep. However, a segregation of the haplotype 2 with a certain horn form was not verified for the tested Icelandic sheep. Surprisingly, none of the analyzed sheep, regardless of the horn phenotype, carried the so-called haplotype 2 (OAR10 29 461 968: T + OAR10 29 462 010: T), which Pan et al. (2018) reported to be common in breeds with curled or spiral horns. In contrast to the sheep used by Pan et al. (2018), no length measurements were available for the examined Icelandic sheep. However, the breeder reported retrospectively that all the sheep homozygous with regard to the T allele (OAR10_29461968) were the ones that developed the "strongest" horns. Furthermore, as the allele T of SNP OAR10_29461968 was mainly present in normally horned sheep, the previously seen connection of increased horn length with the amount of T copies (Pan et al., 2018) seems also to be observed in Icelandic sheep. Furthermore, the breeder noted that, among the oval-horned sheep, the appearance of the horns of the only two sheep homozygous with regard to C was very similar, while the other sheep with

Table 4. An overview of Icelandic sheep of farm no. 2 and the occurrence of the 4 bp deletion in *HOXD1* and the *RXFP2* genotype. All sheep from polycerate families (no. 1–8) carry the 4 bp deletion regardless of whether they are polycerate or polled polycerate. All remaining two-horned or polled sheep originating from farm no. 2 (No. 9–34) did not carry the *HOXD1* variant.

Sample no.	Horn phenotype	Sex	*n*	4 bp deletion in *HOXD1*	*RXFP2* genotype
1	Polycerate (four to six horns)	Female	1	del/–	–/–
2		Female	1	del/–	Failed
3		Male	1	del/–	ins/–
4		Male	1	del/del	Failed
5	Polled polycerate (four to six horns)	Female	1	del/del	–/–
6		Female	1	del/–	ins/–
7		Male	1	del/del	ins/–
8		N/A	1	del/del	ins/–
9–13	Horned (two horns)	Female	5	–/–	–/–
14–24		Male	10	–/–	–/–
25–26		Male	2	–/–	ins/ins
27		Male	1	–/–	ins/–
28		N/A	1	–/–	–/–
29–31	Polled (two horns)	Female	3	–/–	ins/–
32		Female	1	–/–	ins/ins
33–34		Male	2	–/–	ins/ins

N/A – not analyzed.

oval horns (with genotypes CT and TT) differed from these two (Fig. 4).

However, without specific horn length measurements at a certain age for the sheep analyzed, the influence of the SNV OAR10_29461968 cannot be evaluated exactly, but tendencies can be pointed out. Interestingly, Sim and Coltman (2019) could not confirm the mentioned association for Thinhorn sheep. In those, none of the loci mentioned before were significantly associated with horn size, but instead, two other SNVs on chromosome 2 and 3 (OAR2_43601714 and OAR3_134140997, respectively) were shown to be associated with horn length (Sim and Coltman, 2019). One problem of studying a quantitative trait, which is as diverse as horns, is the correct phenotyping for classification, especially when it comes to morphology or horn status in breeds with a variable status and the occurrence of scurs. Therefore, it is also possible that, although the horn shapes show great similarities, they are nevertheless different phenotypes. In such a case, it would not be surprising that no association was found in Icelandic sheep as the transferability of the findings from Pan et al. (2018) would be low.

We were able to confirm the association between the *HOXD1* variant (Allais-Bonnet et al., 2021) and the occurrence of multi-hornedness (polyceraty) in the analyzed Icelandic sheep. Only sheep from the multi-horned flock carried the associated *HOXD1* deletion. In addition, no individual from the polycerate family with only two horns carried it. Interestingly, four polled sheep originating from the multi-horned family showed the 4 bp deletion as well. Until now, this has been observed as a dominant trait when compared with two horns, and we expected to observe multi-hornedness in all sheep carrying the *HOXD1* deletion. No comparison with former results can be made as the sheep analyzed by Allais-Bonnet et al. (2021) and partly also by Greyvenstein et al. (2016) were all phenotyped as polycerate or two-horned or scured – no polled individual was mentioned in these studies. Polledness is not reported to occur in Jacob sheep consistently and is just reported for females in the breeds Navajo-Churro and Damara (Porter et al., 2016).

To the best of our knowledge, this is the first study in which polycerate sheep and polled family members were genotyped simultaneously for the 1.78 kb sized *RXFP2* insertion and the *HOXD1* 4 bp deletion. Based on our results in a low number of samples, it seems that polledness in sheep with the *HOXD1* deletion is not caused by the presence of the *RXFP2* insertion. Notably, even a single polycerate polled ewe carried the *RXFP2* wild type. A further investigation of polledness in sheep carrying the *HOXD1* deletion needs to be conducted with a larger sample set in the future. However, as far as can be hypothesized from the present results, it seems that at least one other variant besides the *RXFP2* insertion controls the absence of horns in polycerate animals. This probably acts epistatically on the *HOXD1* variant, resulting in polled sheep in the presence of the polyceraty allele (4 bp del in *HOXD1*).

A recent study found that genes such as *FOXL2*, *TNN*, and *ACAN*, in addition to the well-known *RXFP2*, are involved in horn development in ovines (Luan et al., 2023). This supports the assumption that other gene variants have an impact on the complex horn phenotype trait. Just recently, a study on

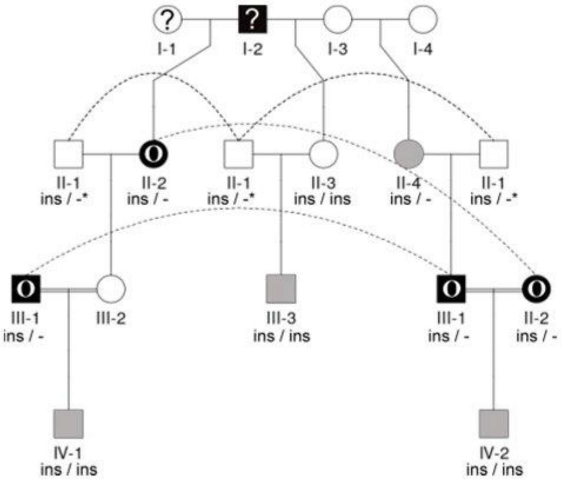

Figure 3. Pedigree displaying the inheritance of horn status in Icelandic sheep genotyped for the *RXFP2* insertion, starting from scured descendants in the current generation and moving backwards. The pedigree shows four generations (I-IV) including available horn status information (gray: scured, white: polled, black with O: oval horns, black with ?: horn form unknown, ?: unknown), *RXFP2* genotype (* indicates suggested genotype), and sex (circle: female, square: male). Dashed lines indicate the same animal in different matings. III-1, III-2, and II-2 are half-siblings, indicated by a double line. Please note that animal III-2 was not available for sampling. Focusing on scured progeny, it is shown that scured males derived from polled parents (III-3), horned (oval) parents (IV-2), and a paring of a horned (oval) father and a polled mother (IV-1).

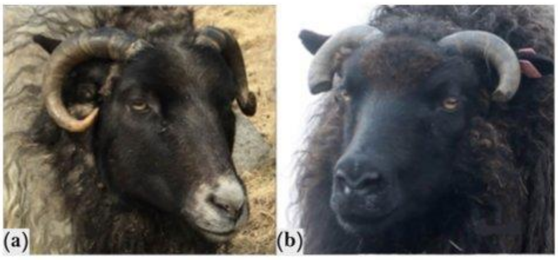

Figure 4. Comparison of two muttons with oval horns originating from farm no. 1. The left sheep **(a)** shows the usual oval horns, while the one on the right-hand side **(b)** shows oval horns that grow sideways towards the face (to avoid injuries, they had to be cut off). The latter was only observed in sheep with OAR10_29461968: CC. Please note that the animals are not exactly the same age; therefore, no comparison of horn size or length should be made.

whole-genome sequences of more than 1000 sheep (representing ∼ 150 breeds and seven wild sheep species) revealed three major haplogroups (hap-a, hap-b, hap-c) in the *RXFP2* region, which were highly frequent in polled, sex-specific, and horned breeds, respectively (Cheng et al., 2023). There is evidence that these haplogroups were introgressed from Iranian mouflon. Nevertheless, it is still possible that all direct ancestors of domestic sheep carried them as well (Cheng et al., 2023). Furthermore, it was postulated that at least hap-c was introgressed before the worldwide spread related to the domestication of sheep (Cheng et al., 2023). However, since no further alleles associated with polledness in sheep have been identified in the meantime (Nicholas and Tammen, 2023b), many questions, especially on the breed-specific and sex-dependent genetic control over the presence or absence of horns, remain unanswered.

5 Conclusions

As in other sheep breeds with variable horn status, the inheritance of horn status (in terms of presence or absence) proved to be complex in Icelandic sheep, especially when sheep carry anything other than regularly formed horns. However, polled × polled matings seem to be a relatively reliable way to produce polled offspring.

To our knowledge, this is the first detailed study of horn status in Icelandic sheep that also includes polyceraty, as well as horn shape, based on already-known variants and markers. Although nearly all polled Icelandic sheep carried the 1.78 kb sized *RXFP2* insertion, at least on one chromosome, and although the majority of regularly horned seep were homozygous with regard to the *RXFP2* wild type, similarly to other sheep breeds with variable horn status, no perfect segregation of this variant with horn status was observed, especially in sheep with scurs and oval horns.

A trend in association was also observed for the previously published link between the SNP OAR10_29461968 (TT), located in the *RXFP2* gene, and increased horn length in Icelandic sheep.

The interplay of polyceraty, which segregated perfectly with the published 4 bp deletion in *HOXD1* in Icelandic sheep, and polledness should be investigated in more detail on a larger sample set and by also taking into account other variants besides the 1.78 kb sized *RXFP2* insertion.

As an isolated population with extensive information about the individual animal, the Icelandic sheep provide a promising basis for further investigations considering horn-status-related and other traits, as well as for diversity analyses. Follow-up investigations will be needed for larger sample sets, with more detailed information on horn morphology, and these should also make use of techniques that have been further developed in the meantime, such as long-read sequencing, to address potentially more involved, complex variants.

Data availability. For detailed information on the data, please refer to Table S3.

Supplement. The supplement related to this article is available online at: https://doi.org/10.5194/aab-67-237-2024-supplement.

Author contributions. Conceptualization: RS, GL. Formal analysis: RS. Investigation: RS. Resources: KE, GL. Supervision: GL. Visualization: RS. Writing – original draft preparation: RS, GL. Writing – review and editing: KE, GL, RS.

Competing interests. The contact author has declared that none of the authors has any competing interests.

Ethical statement. All samples used in this study were taken for the purpose of diagnostics and forwarded to us for additional use afterwards. Sample collection was performed by trained personnel and under standards of good professional practice.

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4. DISCUSSION

The genetic factors influencing various horn traits exhibit considerable variation across bovid species, but also within species. In the context of livestock keeping, polledness is a particularly interesting characteristic.

Horned livestock can pose an increased risk of injury to both flockmates and handlers (Braun et al., 2016; Menke et al., 1999). Injuries among the animals can lead to economic losses, for example due to reduced milk quality and quantity (Mendonça et al., 2016; Youngers et al., 2017). From a broad perspective, there are three different approaches to address this issue. Recommendations for adjustments in management and the husbandry environment (Aschwanden et al., 2009, 2008; Hillmann et al., 2014; Loretz et al., 2004), which cannot be implemented universally for various reasons. The practice of mechanical dehorning / disbudding, as a second approach, is increasingly under criticism in the context of the intensified animal welfare debate. For example, the dehorning of goat kids is already completely prohibited by law in Germany (Deutscher Bundestag, 2006). The approach of genetic polledness and the associated breeding and selection in this area is considered an animal-friendly alternative. What is practically and largely unproblematic in the cattle sector (Prayaga, 2007) – the genetic basis is known for various cattle breeds and no severe associations are known – leads to problems in goat breeding.

According to Asdell's 1944 description, the connection between the dominant trait of polledness and the recessive trait of intersexuality in goats results in homozygous polled female goats being intersexual (also known as "polled-intersexes"), expressed in an variable phenotypic extent (Asdell, 1944). This inhomogeneity of the phenotype leads to difficulties in the early detection of affected animals in practice. Subsequently this leads to economic losses and reduced breeding success (infertility of polled-intersexes). The development of genetic testing based on the already published 11.7 kb-sized insertion on chromosome 1 (Pailhoux et al., 2001; Zhang et al., 2020) failed for European breeds. Although Zhang et al. (2020) reported that differentiation of all three possible genotypes for the PIS mutation was possible in their study. Using long-read whole genome sequencing, a much more complex variant was found that is linked to the already known insertion (Simon et al., 2020). This now known to be smaller ~ 10 kb-sized insertion, combined with an inversely inserted ~ 480 kb-sized duplicated segment of a region downstream of chromosome 1 has been confirmed several times for other breeds since this first publication (E et al., 2020; Guo et al., 2022).

With reference to the new findings, not only the two genes *PISRT1* and *FOXL2*, but also *potassium inwardly rectifying channel subfamily J member 15* (*KCNJ15*) and *ETS transcription factor ERG* (*ERG*) are potentially influenced by the observed variant. Copy number variants in *KCNJ15* were just recently shown to significantly correlate with growth traits in four out of five analysed Chinese goat breeds (Zhao et al. 2024). Furthermore, it has been shown that the published complex variant leads to an interchromosomal rearrangement and the formation of loop structures of chromosome 1 in the region of the *FOXL2* gene (E et al., 2020). The assumption that this structural change leads to an altered expression of *FOLX2* or neighboring genes still needs to be verified (E et al., 2020). The role of *FOXL2* in mammalian sex determination is undisputable (Migale et al., 2021) and it has already been shown that its loss of function leads to female-to-male sex reversal in goats (Boulanger et al., 2014). A recent gene expression study indicates that various pathways and thereby various physiological systems are involved in the development of intersexual goats (Han et al., 2022). However, it is important to note that the cause of intersexuality in those analyzed Huai goats (Han et al., 2022) has not been fully explained; As such, any direct link to PIS should be viewed with caution.

However, looking at the polled/horned trait in goats independently, a recent publication investigating more than 300 genes showed an association in the comparison of whole genome data from 31 polled individuals (three breeds) and 15 horned individuals (one breed). *Stromal interaction molecule 1* (*STIM1*) on chromosome 15 and *neurexin 1* (*NRXN1*) on chromosome 11 were stated as possible candidate genes for the horned phenotype in goats (Wan et al., 2023).

Information found on individual breeds in which the association between polledness and intersexuality is not supposed to occur could neither be confirmed by literature research nor, in the case of Maltese goats, by carrying out the genetic test developed by Simon et al. (2020). In general, the described variant is present in all polled individuals examined, but not in horned individuals. It can therefore be assumed that all breeds worldwide are equally affected by PIS, caused by the same complex variant, and the associated challenges influence breeding for polledness in this species.

In cattle, the Celtic polled (Medugorac et al., 2012) variant has already been successfully integrated/inserted into a horned bulls fibroblasts genome by genome editing, thus producing polled offspring (Schuster et al., 2018; Schuster et al., 2020). Already ahead, attempts were successful which used other endonucleases, e.g. TALEN (Tan et al., 2013). No information is available on whether attempts have been made to use one of the known polled variants in one of the species to edit in the presence or absence of horn in another species. For instance, it could be tested if inserting the Celtic polled variant from cattle, ~200 bp-sized, into the genome of a horned goat leads to the expression of the desired trait without side effects (intersexuality) in the target species, for example using the clustered regularly interspaced short palindromic repeats (CRISPR) system (Jinek et al., 2012). Another interesting aspect in addition to generating polled goats without associated intersexuality, would be to investigate whether only one of the two combined variants (Simon et al., 2020) leads to polledness, and if so which one. For cattle, a similar study was recently published (Hennig et al., 2022). It was shown that the mere 10 bp deletion, which is replaced by a 212 bp duplication of a DNA segment in the Celtic variant, does not alone lead to polledness. Only the combination of deletion and duplication leads to the lack of horn bud development (Hennig et al., 2022).

However, it remains debatable whether this genome editing methods, if successful, would have any practical use for breeding polled goats. European legislation at least classifies CRISPR technology as genetic engineering (EuGH, 2018), which makes its use in food producing animals challenging. In addition, consumer concerns must be seen as a limiting factor (Canavari and Nayga, 2009), even if the potential of genome editing remains undisputed (Wang and Doudna, 2023; van Eenennaam, 2019). In the field of gene-edited plants, there has been a recent push to change European regulations (Vanderschuren et al., 2023; Nature Plants E, 2023). The European Parliament voted to ease the regulation of gene-edited crops, applying to changes that could also have been achieved by conventional breeding (European Parliament, 2024).

However, by the new findings the development of a genetic testing for PIS was finally possible and thereby already offers valuable implications for the management and breeding of polled goats (Simon et al., 2020). It is important to note that intersexuality in goats can occur independently of polledness as well. XX/XY chimerism (freemartinism), as recently described by Paredes et al. (2024) in one of two case reports, is one of the causes (Paredes et al., 2024). Cases like these cannot be detected using the mentioned genetic testing.

Comparing the current state of knowledge on the genetic factors of polledness in cattle, goats, and sheep reveals two observations (Simon et al., 2022). First, the variety of genetic factors involved for a trait (polledness) that hardly differs between the species phenotypically. Interestingly studies found that the two genes *FOXL2* and *RXFP2* seem to be the only ones, to a different extent, which are involved in polledness in all three species (Simon et al., 2022). The second conspicuous feature is the fact that there are still major gaps in knowledge and this applies to sheep in particular.

After it was shown that the ~1.8 kb-sized *RXFP2* variant published nearly a decade ago (Wiedemar and Drögemüller, 2015) does not segregate with polledness in several breeds with variable horn status (Lühken et al., 2016), no further variants or candidate genes could be found in sheep (Nicholas and Tammen, 1995). The influence of *RXFP2* on the horn bud development in sheep was confirmed by a recent study, that furthermore identified few other genes, such as *SFRP4* and *WNT3*, which are involved as well (Luan et al., 2023). However, not only the genetic factors of polledness in sheep still raise questions, but also the inheritance of this trait has not been clarified across the species (N.K. Pickering, P.L. Johnson, B. Auvray, K.G. Dodds, J.C. McEwan, 2009; Clutton-Brock and Pemberton, 2004; Johnston et al., 2011). Other horn associated traits such as size, shape or number are highly variable in sheep as well. Associated variants or markers are often only described for individual breeds (Johnston et al., 2010; Pan et al., 2018; Duijvesteijn et al., 2018). To account such observed or expected breed differences the scientific consideration of new breeds can be helpful (Marshall, 1994; Salonen et al., 2019; Aldersey et al., 2020). One breed in which both polledness and variations in horn size and shape, as well as the characteristics of scurs and polyceraty occur are Icelandic sheep (Dýrmundsson and Niżnikowski, 2010). Detailed records about phenotypes and relationship, as the basis for animal breeding and genetic research (Seidel et al., 2020), are available through the breeders. These facts and the intensive isolation of the breed due to import restrictions caused by eradication programs in recent decades and the isolated location of Iceland make it an interesting breed to verify the findings published so far.

The recent study of Simon et al. (2024) therefore fulfilled the first step to include the Icelandic sheep breed into the research field of horn associated traits (Simon et al., 2024). The results reflect the diversity of the characteristics associated with horns in sheep and the variability of previous publications and results (in the sense of not being generally valid). The 1.78 kb-sized *RXFP2* variant associated with polledness was found in the population, but, as expected, there was no perfect segregation with the horn status (absence / presence of horns) (Simon et al., 2024). It is possible that there is a similarity to cattle (Medugorac et al., 2012; Medugorac et al., 2017), in which different variants are known to be independently associated with the same trait polledness. Which breeds could cluster and on which basis, however, remains unclear in sheep and offers potential for further research. The same might apply for scurs in sheep, in which the association with the described *RXFP2* variant was week (Simon et al., 2024).

Observed tendencies of an influence of *RXFP2* on horn size (Pan et al., 2018), which was also visible in Icelandic Sheep (Simon et al., 2024) once again indicates the great influence of this gene on horn-associated traits in sheep. In addition, just recently it was published that a *RXFP2* haplotype, associated with spiral horn form in the wild species, was passed on from Iranian mouflons into different sheep breeds worldwide and contributed to their morphological differentiation (Cheng et al., 2023). A link between horn shape and another, already published marker in the *RXFP2* gene (Pan et al., 2018) could not be confirmed in the analyzed Icelandic sheep (Simon et al., 2024). However, it is difficult to compare horn shapes of different breeds based on pictures. The standardized measurement of specific characteristics, such as the perimeter of the horn at the base of the horn, taking into account the age of the animal, can help to increase comparability, but at the same time complicate data collection in the field.

Two completely new aspects that are connected with polyceraty and the associated 4 bp deletion in *HOXD1* (Allais-Bonnet et al., 2021; Zhang et al., 2023) were also observed in the Icelandic sheep (Simon et al., 2024). The described variant was, in contrast to all analysed breeds until now, also present in phenotypically polled individuals deriving from polycerate families. In addition, it is apparent that polledness in these individuals is not controlled by the *RXFP2* insertion (Simon et al., 2024). It would be important to know whether this applies to a larger sample set of Icelandic sheep, as well as other polycerate breeds. A closer look at these phenotypically polled animals with polycerate origin could also be a new starting point for further identification of additional variants associated with polledness in sheep. Literature review revealed that this has not been focused yet.

There was no difference in the occurrence of *HOXD1* variant between four- and six-horned sheep. Whether a further, possibly linked but yet unknown variant codes for the final number of horns is still unknown. However, it seems certain that the "supernumerous" horns also always occur in pairs. The breeders or owners were able to confirm that the horns had grown together in all cases where an uneven number of horns was initially observed (Figure 4).

Taken together, the results once again confirm the assumption of a strong breed influence and the important role of the *RXFP2* gene with regard to the investigated horn-associated traits in sheep, excluding polyceraty.

Figure 4: Polycerate icelandic ewe.
Please note that the two left horns fused (red arrow) while the right ones are clearly separated in two horns (picture: María Fríðgerður Bjarnadóttir).

4.1. Future studies – outlook

As still a lot of aspects of the topic horns / absence of horns remain unknown, this leaves space for further research particularly in sheep and goats. In the light of new techniques or their advancements there is a chance to solve long persisting problems, like shown in the current work for PIS. Even if PIS is already known since the 1940`s and it has been widely reported that the resulting phenotype is highly variable, the effects on the hormonal status of affected individuals, the exact phenotypic expressions in female homozygous hornless animals, and the influence of PIS on fertility in male goats remain largely unexplored. Necessary studies should aim to answer long unanswered questions, primarily whether female homozygous polled goats are sterile in every case and if so, if there is a way to solve that problem.

4.2. Conclusion

With the findings of this work it was possible to proof previous publications and extend the knowledge in the field of genetic factors in horn status trait. With this, the findings especially on the polled intersex syndrome and the variant for polyceraty in Icelandic sheep highlighted new aspects and laid the groundwork for subsequent investigations to unravel the underlying genetic mechanisms of horn traits in small ruminants. Once again the findings proved the multifaceted nature of the genetic architecture governing these traits, especially in sheep. Updated information were summarized about polledness in sheep and goats including cattle, which before was often seen apart from each other. The work thereby offers valuable implications for a better understanding of genetic factors influencing horn status traits in bovidae and contributes an up to date summary of the body of knowledge in the field of genetic polledness. The present and future progress in molecular genetics gives reason to hope that also the black spots of the complex trait of polledness in sheep can be gradually filled with knowledge, which will help to provide targets for selective breeding for traits of interest or even genome editing.

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Appendix

First publication – supplementary files

The supporting information can also be found online:

https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1111%2Fage.12918&file=age12918-sup-0001-Supinfo.docx

Table S1. Horn status and PIS genotypes of 814 goats from 23 breeds.

Breed	**Genetic sex**	**Status**		
		horned	**heterozygous polled**	**homozygous polled (resp. PIS in XX)**
Chamois Colored	XY	8	4	
	XX	44	21	
Saanen Goat	XY	4	10	2
	XX	39	49	4
Toggenburg Goat	XY	16	14	
	XX	57	21	
Grisons Striped Goat	XY	14	5	1
	XX	104	27	1
Appenzell Goat	XY		17	2
	XX	11	41	1
Boer Goat	XY	1	2	
	XX	14	3	3
Valais Blacknecked Goat	XY	2		
	XX	19		
Booted Goat	XY	1		
	XX	21		
Peacock Goat	XY	1		
	XX	28		
Nera Verzasca Goat	XY			
	XX	19		
Tessin Grey Goat	XY			
	XX	14		
Thüringerwald Goat	XY	1	1	1
	XX	13	10	2
Damascus	XY	1	2	
	XX			
Baladi	XY	5	1	
	XX	7	4	
Zaraibi	XY	11	1	
	XX	11	2	
Maltese	XY			
	XX	4	6	
Weisse Deutsche Edelziege	XY		4	1
	XX		10	
Bunte Deutsche Edelziege	XY	1	12	2
	XX		5	2
Syria	XY	1		
	XX	1	2	
Anglo-Nubian	XY	1	2	
	XX		3	1
Czech White Short-haired Goat (Bílá kratkosrsta)	XY			
	XX	3	23	
Czech Brown Short-aired Goat (Hnedá kratkosrsta)	XY			
	XX	3	8	
Bulgarian Goat	XY			
	XX			1

Table S2. Output data of long-read whole-genome sequencing.

Sample		Nb reads	Min length [bp]	Max length [bp]	Average length [bp]	Total length [Gbp]	Theoretical genome coverage (goat ASR1 assembly)
PIS-affected (SAN096)		960936	134	135619	5317.98	5.11	
		1611709	92	277611	2695.4	4.34	
		1156181	93	243967	3383.9	3.91	
		1524079	113	259429	3134.53	4.78	
		432	148	97927	8000.71	3.46	
		524132	113	178739	4088.48	2.14	
		363264	148	173779	6840.47	2.48	
	Total	**6572301**	**92**	**277611**	**3990.85**	**26.23**	**8.97**
horned control (VAG203)		858085	118	147283	8411.66	7.22	
		907693	165	139622	7860.28	7.13	
		753112	135	222491	6705.39	5.05	
		284818	228	132399	10153.63	2.89	
		136897	85	136897	9384.37	8.36	
		573473	135	224719	8273.32	4.74	
		800278	108	168785	6954.94	5.57	
	Total	**5068569**	**85**	**224719**	**8079.28**	**40.97**	**14.02**

Table S3. Whole-genome short-read goat sequences.

Sample ID	Sex	Breed	Genotype	Coverage	Study accession	Sample accession
BST131	m	Grisons Striped Goat	homozygous polled	23.1	PRJNA310684	SAMN07551950
SAN081	m	Saanen Goat	homozygous polled	25.6	PRJNA310684	SAMN04453660
39183_DNA	f	Saanen Goat	heterozygous polled	13.8	PRJNA310684	SAMN09841864
40613_DNA	f	Saanen Goat	heterozygous polled	9.8	PRJNA310684	SAMN09841865
SAN041	f	Saanen Goat	heterozygous polled	13.8	PRJNA310684	SAMN04453657
SAN049	f	Saanen Goat	heterozygous polled	31.2	PRJNA310684	SAMN04453658
SAN075	f	Saanen Goat	heterozygous polled	14.3	PRJNA310684	SAMN04453659
37969_DNA	f	Chamois Colored Goat	control, horned	16.8	PRJNA310684	SAMN09841859
38017_DNA	f	Valais Blacknecked Goat	control, horned	19.1	PRJNA310684	SAMN09841860
38212_DNA	m	Dwarf Goat	control, horned	11.5	PRJNA310684	SAMN09841861
38334_DNA	f	Crossbred	control, horned	17.5	PRJNA310684	SAMN09841862
38563_DNA	f	Crossbred	control, horned	15.1	PRJNA310684	SAMN09841863
41567_DNA	m	Saanen Goat	control, horned	17.4	PRJNA310684	SAMN09841866
BST001	f	Grisons Striped Goat	control, horned	9.6	PRJNA310684	SAMN04453620
BUR09	f	Boer Goat	control, horned	15.6	PRJNA310684	SAMN04453621
BUR29	f	Boer Goat	control, horned	11	PRJNA310684	SAMN04453622
CAG038	m	Tessin Grey Goat	control, horned	11.6	PRJNA310684	SAMN04453623
GFG024	f	Chamois Colored Goat	control, horned	9.9	PRJNA310684	SAMN04453654
GFG034	f	Chamois Colored Goat	control, horned	11.7	PRJNA310684	SAMN04453655
KHZ098	f	Valais Coppernecked Goat	control, horned	11.7	PRJNA310684	SAMN04453656

Table S4. PCR primers for genotyping and determination of horn and sex status.

Allele	Primer name	Product size	Sequence 5'-->3'	Strand	Start position (goat ASR1 assembly)	Remarks
Chr 1 wild type	Goat_PIS_F1		TCATAGGCCATAGCTAAATGGT	forward	Chr 1: 129424628	used in diagnostic multiplex PCR
(horn)	Goat_PIS_Rwt	398	AATGCAGGGGTTGAGGATTT	reverse	Chr 1: 129425025	used in diagnostic multiplex PCR
Chr 1 variant	Goat_PIS_F2		GACCATCACAGTGTCTGACTTGA	forward	Chr 1: 150334394	used in diagnostic multiplex PCR
(polled - PIS)	Goat_PIS_R2	143	TTTGTTCGTAGTGACAATACTGG	reverse	Chr 1: bridging the fusion of the breakpoints (129434939/150334286)	used in diagnostic multiplex PCR
Chr 1 variant	Goat_PIS_F1		TCATAGGCCATAGCTAAATGGT	forward	Chr 1: 129424628	bridging the breakpoints
(polled - PIS)	Goat_PIS_R1	272	GAGACAGGCTGAATGTGCAA	reverse	Chr 1: 150817981	bridging the breakpoints
Chr X and Y	AMELXY		AGCAACAGACAAGACCAAGC	forward	Chr X: 58862859 NW_017189585.1:80093	used in diagnostic multiplex PCR
Chr X	AMELX	245 (264 according to Tsai et al. (2011))	CAATAGCTTTTATGGTGG	reverse	Chr X: 58862615	used in diagnostic multiplex PCR
Chr Y	AMELY	206	GCTTGTCTATCTATATGGCA	reverse	NW_017189585.1:80298	used in diagnostic multiplex PCR

Figure S1. Nanopore long-read sequencing details. (a) IGV screenshots of long-read sequences data showing the 10.1 kb deletion at ~129 Mb and the ~480 kb duplication at ~150 Mb in the PIS-affected goat in comparison to a horned goat. (b+d) Close-ups of IGV screenshots showing soft-clipped long-read sequences at the boundaries of the variant sites. (c) Circos plot showing the split-mapped reads across the breakpoints. The coverage is shown in blue in the outer circle for both involved genome regions of chromosome 1.

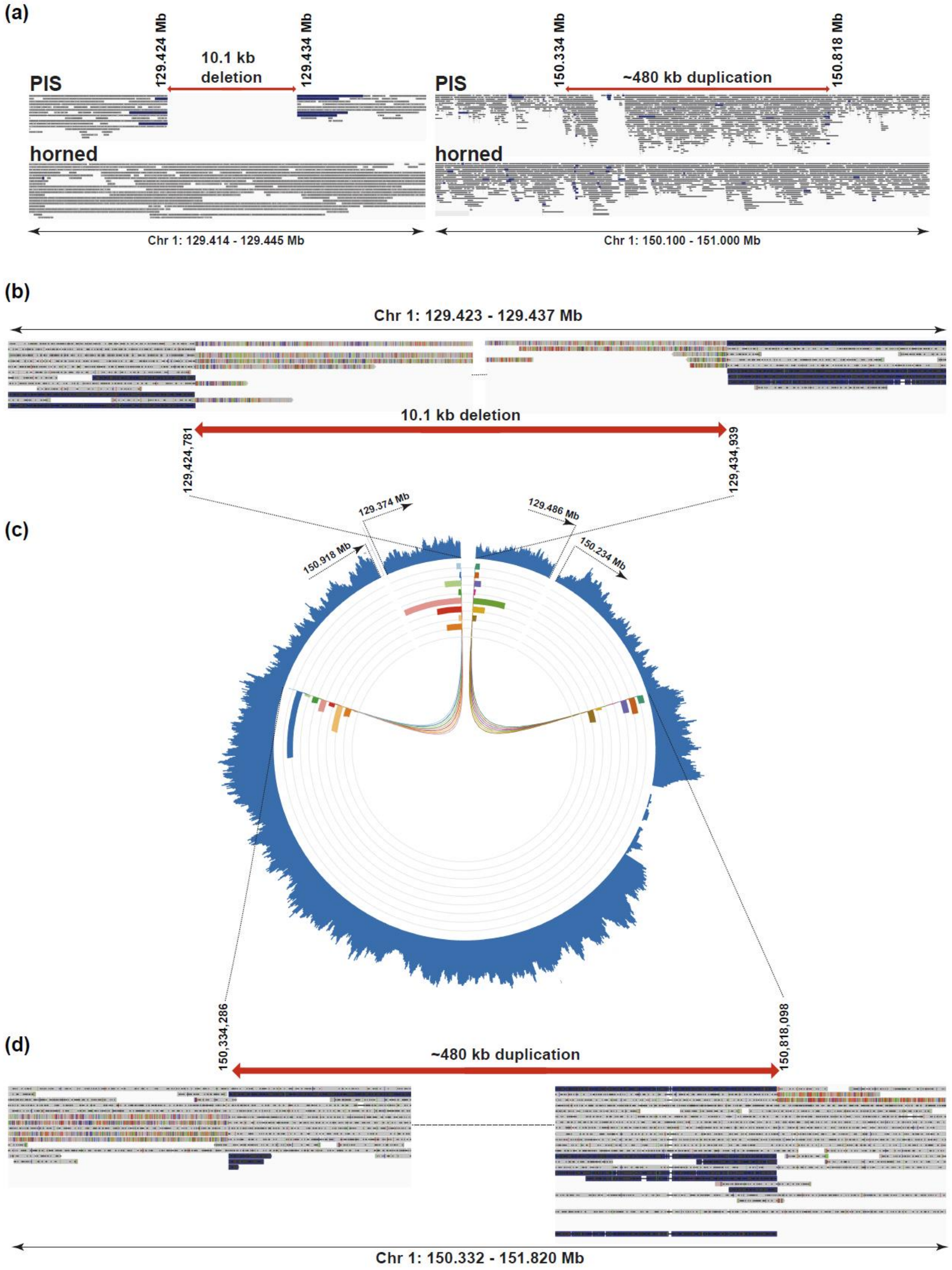
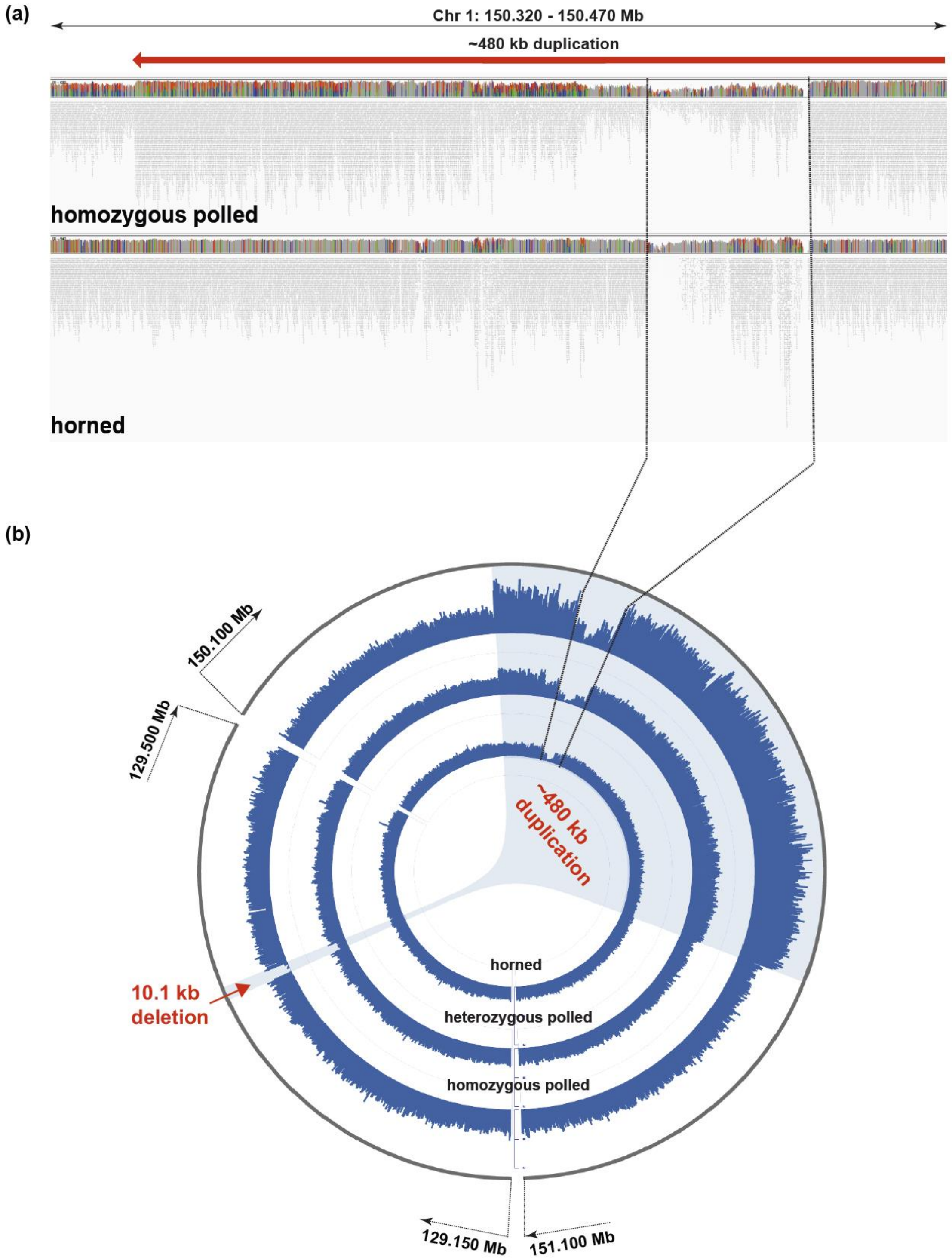


Figure S2. Illumina short-read sequencing details. (a) IGV screenshots of short-read sequences data showing a ~57 kb region with low mapping quality within the duplicated segment. This is seen in all animals for this region indicating a discrepancy with the reference on goat chromosome 1. (b) Circos plot showing the average short-read sequence depth of sequenced goats for the chromosome 1 segment containing the deletion at ~129 Mb and the region with the duplication at ~150 Mb. The inner circular track shows the average coverage for 13 horned, the central circular track for 5 heterozygous polled, and the outer circular track for 2 homozygous polled goats.



Second publication – supplementary files

The supporting information can also be found online:

https://www.mdpi.com/article/10.3390/genes13050832/s1

Table S1. Whole genome sequencing data from Dorper sheep. The data derived from short-read sequening and is publically available via the European Nucleotide Archive (ENA). All nine animals are males.

Accession Number	Breed	Horn status
SAMEA6531511	Dorper Sheep	horned
SAMEA6531507	Dorper Sheep	horned
SAMEA5720682	Dorper Sheep	horned
SAMEA6531512	Dorper Sheep	horned
SAMEA5720681	Dorper Sheep	horned
SAMEA6531508	Dorper Sheep	scured
SAMEA6531509	Dorper Sheep	scured
SAMEA5720683	Dorper Sheep	polled
SAMEA6531510	Dorper Sheep	polled

Table S2. Sheep genotyped with genetic testing for polled intersex syndrome in goats. Samples of different sheep breed genotyped with the two existing version of the PIS genetic testings. Simon et al. (2020) established a multiplex PCR combining the determination of the sex and the horn status. In Guo et al. (2021) a 369 bp sized PCR product indicates a horned animal, while a PCR product of 1822 bp length is just visible in polled goats.

Breed	Sex	Horn status	PIS genetic testing (Simon et al. 2020)	PIS genetic testing (Guo et al. 2021)
Merinoland Sheep	male	polled	male, horned	horned
	female	polled	female, horned	horned
African Dorper	male	polled	male, horned	horned
	male	horned	male, horned	horned
	male	scured	male, horned	horned
	female	polled	female, horned	horned
Kerry Hill Sheep	male	polled	male, horned	horned
	female	polled	female, horned	horned
Rhone Sheep	male	polled	male, horned	horned
	female	polled	female, horned	horned
Barbados Blackbelly Sheep	male	polled	male, horned	horned
	female	polled	female, horned	horned
Cameroon Sheep	male	horned	male, horned	horned
	female	polled	female, horned	horned
East Friesian Milk Sheep	male	polled	male, horned	horned
	female	polled	female, horned	horned
Texel Sheep	male	polled	male, horned	horned
	female	polled	female, horned	horned
Soay Sheep	male	n/a	male, horned	horned
Mouflon	n/a	n/a	male, horned	horned
Lacaune Sheep	male	polled	male, horned	horned
	female	polled	female, horned	horned
Scottish Blackface Sheep	female	horned	female, horned	horned
Ouessant Sheep	male	horned	male, horned	horned
	female	polled	female, horned	horned
Roughwooled Pommeranian Sheep	male	polled	male, horned	horned
	male	scured	male, horned	horned
Krainer Steinschaf	female	n/a	female, horned	horned
Grey Horned Heath	n/a	horned	male, horned	horned

Third publication – supplementary files

The supporting information can also be found online:

https://doi.org/10.5194/aab-67-237-2024-supplement

Table S1 Genotyping results for the previously published SNPs OAR10_29 461 968 and OAR10_29 462 010 that showed an influence on horn length and form in Chinese breeds (Pan et al. 2018) in selected Icelandic sheep with the displayed horn phenotypes. Please note that for the latter SNP just the C allele was present in the analysed sample set.

		OAR10_29 461 968			OAR10_29 462 010
Horn phenotype	**Sex**	**C / C**	**T / C**	**T / T**	**C / C**
polled	female	1	1		2
	male				
normal horned	female		1	1	2
	male		3	4	7
	N/A			1	1
scurs	female		6		6
	male		3		3
oval horns	female			1	1
	male	2	9		11
polycerate (4-6 horns)	female			1	1
	male		1	1	2
polled polycerate (4-6 horns)	female		1	1	2
	male		1		1
	N/A		1		1

Table S2 Horn status of the offspring from various matings on farm N°1, indicating which horn status derived from which parent combination. Please note that for some parents no detailed horn information was available, indicated by horns (?).

			Horn status progeny			
Mating of different horn status parents			**Polled**	**Normal horned**	**Scurs**	**Oval horns**
polled	X	polled	12		2	1
polled	X	scurs	5			3
polled	X	normal horns	2	4		2
polled	X	oval horns	8		4	
normal horns	X	normal horns	1			1
normal horns	X	oval horns		1		
normal horns	X	scurs		2		
oval horns	X	oval horns	1		1	2
oval horns	X	scurs	3			3
polled	X	horns (?)	2			
scurs	X	horns (?)			1	
?	X	polled	1			
?	X	horns (?)			1	1
?	X	scurs		2		
?	X	?	3		1	

Table S3 Details on all Icelandic sheep used for the analysis. Please note that the individuals used for genotyping of the different variants are marked.

farm	lab N°	sex	horn status	details on horn from or number	details on mothers horn phenotypes	details on fathers horn phenotypes	*RXFP2* insertion (Wiedemar and Drögemüller (2015))	OAR10_29458450 (Dujivesteijn et al. (2018))	OAR10_29461968 ("Pan5", Pan et al. (2018))	OAR10_29462010 ("Pan6", Pan et al. (2018))	*HOXD1* deletion (Allais-Bonnet et al. (2021))
N°1	20224504	female	horns	normal, 2	scurs	unkown	✓	✓	✓	✓	✓
N°1	20213679	female	horns	normal, 2	scurs	horns, normal, 2	✓	✓	✓	✓	✓
N°1	20223748	male	horns	normal, 2	scurs	unkown	✓	✓	✓	✓	✓
N°1	20213677	male	horns	normal, 2	horns, oval	horns, normal, 2	✓	✓	✓	✓	✓
N°1	20223678	male	horns	normal, 2	scurs	horns, normal, 2	✓	✓	✓	✓	
N°1	20213683	male	horns	normal, 2	polled	horns, normal, 2	✓	✓	✓	✓	
N°1	20213682	male	horns	normal, 2	polled	horns, normal, 2	✓	✓	✓	✓	
N°1	20213680	male	horns	normal, 2	polled	horns, normal, 2	✓	✓	✓	✓	
N°1	20213681	male	horns	normal, 2	polled	horns, normal, 2	✓	✓	✓	✓	
N°1	20214213	female	horns	oval	unkown	horns, no details	✓		✓	✓	✓
N°1	20223773	male	horns	oval	polled	polled	✓		✓	✓	✓
N°1	20223749	male	horns	oval	scurs	polled	✓		✓	✓	
N°1	20223764	male	horns	oval	scurs	unkown	✓		✓	✓	
N°1	20223776	male	horns	oval	horns, oval	horns, oval	✓		✓	✓	
N°1	20223771	male	horns	oval	horns, oval	horns, oval	✓		✓	✓	
N°1	20223766	male	horns	oval	scurs	horns, oval	✓		✓	✓	
N°1	20223751	male	horns	oval	scurs	horns, oval	✓		✓	✓	
N°1	20223768	male	horns	oval	scurs	horns, oval	✓		✓	✓	
N°1	20211430	male	horns	oval	polled	horns, normal, 2	✓		✓	✓	

Table S3 Details on all Icelandic sheep used for the analysis. Please note that the individuals used for genotyping of the different variants are marked. (continued)

farm	lab N°	sex	horn status	details on horn from or number	details on mothers horn phenotypes	details on fathers horn phenotypes	*RXFP2* insertion (Wiedemar and Drögemüller (2015))	OAR10_29458450 (Dujivesteijn et al. (2018))	OAR10_29461968 ("Pan5", Pan et al. (2018))	OAR10_29462010 ("Pan6", Pan et al. (2018))	*HOXD1* deletion (Allais-Bonnet et al. (2021))
N°1	20211431	male	horns	oval	polled	horns, normal, 2	✓		✓	✓	
N°1	20223745	male	horns	oval	horns, normal, 2	horns, normal, 2	✓	✓	✓	✓	
N°1	deceased*	male	horns	oval	scurs	polled	✓				
N°1	20213950	female	polled		polled	horns, no details	✓	✓			
N°1	20213949	female	polled		scurs	polled	✓	✓			
N°1	20213956	female	polled		unkown	unkown	✓	✓			
N°1	20213955	female	polled		unkown	unkown	✓	✓			
N°1	20223750	female	polled		polled	polled	✓	✓			✓
N°1	20223756	female	polled		scurs	polled	✓	✓			✓
N°1	20213954	female	polled		polled	polled	✓	✓			
N°1	20223757	female	polled		polled	polled	✓	✓			
N°1	20223775	female	polled		polled	polled	✓	✓			
N°1	20224503	female	polled		polled	polled	✓	✓			
N°1	20223760	female	polled		polled	polled	✓	✓			
N°1	20223767	female	polled		scurs	horns, oval	✓	✓			
N°1	20223772	female	polled		polled	horns, oval	✓	✓			
N°1	20223765	female	polled		polled	horns, oval	✓	✓			
N°1	20223774	female	polled		polled	horns, oval	✓	✓			
N°1	20223779	female	polled		polled	horns, oval	✓	✓			
N°1	20223753	female	polled		polled	polled	✓	✓			
N°1	20223763	female	polled		polled	polled	✓	✓			
N°1	20223755	female	polled		scurs	horns, oval	✓	✓			

Table S3 Details on all Icelandic sheep used for the analysis. Please note that the individuals used for genotyping of the different variants are marked. (continued)

farm	**lab N°**	**sex**	**horn status**	**details on horn from or number**	**details on mothers horn phenotypes**	**details on fathers horn phenotypes**	***RXFP2* insertion (Wiedemar and Drögemüller (2015))**	**OAR10_29458450 (Dujivesteijn et al. (2018))**	**OAR10_29461968 ("Pan5", Pan et al. (2018))**	**OAR10_29462010 ("Pan6", Pan et al. (2018))**	***HOXD1* deletion (Allais-Bonnet et al. (2021))**
N°1	20223747	female	polled		scurs	horns, oval	✓	✓			
N°1	20223770	female	polled		horns	polled	✓	✓			
N°1	20213952	female	polled		polled	unkown	✓	✓			
N°1	20223762	female	polled		polled	polled	✓	✓			
N°1	20211428	female	polled		polled	horns, normal, 2	✓	✓			
N°1	20211429	female	polled		polled	horns, normal, 2	✓	✓			
N°1	20223746	female	polled		horns, normal, 2	horns, normal, 2	✓	✓	✓	✓	✓
N°1	deceased*	female	polled		horns, oval	polled	✓				
N°1	deceased*	female	polled		horns, oval	polled	✓				
N°1	deceased*	female	polled		scurs	polled	✓				
N°1	deceased*	female	polled		polled	polled	✓				
N°1	deceased*	female	polled		horns, oval	horns, oval	✓				
N°1	deceased*	female	polled		scurs	polled	✓				
N°1	20223758	male	polled		polled	polled	✓	✓			✓
N°1	20223754	male	polled		scurs	polled	✓	✓			✓
N°1	20223777	male	polled		polled	horns, oval	✓	✓			
N°1	20223778	male	polled		polled	horns, oval	✓	✓			
N°1	deceased*	male	polled		polled	polled	✓				
N°1	20221644	male	polled		unkown	unkown	✓	✓			
N°1	20222931	female	scurs		polled	horns, no details	✓		✓	✓	
N°1	20213951	female	scurs		unkown	horns, no details	✓		✓	✓	

Table S3 Details on all Icelandic sheep used for the analysis. Please note that the individuals used for genotyping of the different variants are marked. (continued)

farm	lab N°	sex	horn status	details on horn from or number	details on mothers horn phenotypes	details on fathers horn phenotypes	*RXFP2* insertion (Wiedemar and Drögemüller (2015))	OAR10_29458450 (Dujivesteijn et al. (2018))	OAR10_29461968 ("Pan5", Pan et al. (2018))	OAR10_29462010 ("Pan6", Pan et al. (2018))	*HOXD1* deletion (Allais-Bonnet et al. (2021))
N°1	20214317	female	scurs		unkown	unkown	✓		✓	✓	
N°1	20220500	female	scurs		polled	horns	✓		✓	✓	✓
N°1	20223759	female	scurs		polled	horns	✓		✓	✓	
N°1	20223744	female	scurs		scurs	horns, normal, 2	✓	✓	✓	✓	✓
N°1	20223761	male	scurs		horns, oval	horns, oval	✓		✓	✓	✓
N°1	20223769	male	scurs		polled	polled	✓		✓		
N°1	20223752	male	scurs		polled	horns, oval	✓		✓		
N°1	deceased*	male	scurs		polled	polled	✓				
N°2	20223040	male	horns	polycerate origin, unknown			✓				✓
N°2	20223041	female	horns	polycerate origin, 2			✓				✓
N°2	20223050	female	horns	polycerate origin, 2			✓				✓
N°2	20223059	female	horns	polycerate origin, 2			✓				✓
N°2	20223035	male	horns	polycerate origin, 2			✓	✓			✓
N°2	20223036	male	horns	polycerate origin, 2			✓	✓			✓
N°2	20223037	male	horns	polycerate origin, 2			✓				✓
N°2	20223038	male	horns	polycerate origin, 2			✓				✓
N°2	20223039	male	horns	polycerate origin, 2			✓				✓

Table S3 Details on all Icelandic sheep used for the analysis. Please note that the individuals used for genotyping of the different variants are marked. (continued)

farm	lab N°	sex	horn status	details on horn from or number	details on mothers horn phenotypes	details on fathers horn phenotypes	*RXFP2* insertion (Wiedemar and Drögemüller (2015))	OAR10_29458450 (Dujivesteijn et al. (2018))	OAR10_29461968 ("Pan5", Pan et al. (2018))	OAR10_29462010 ("Pan6", Pan et al. (2018))	*HOXD1* deletion (Allais-Bonnet et al. (2021))
N°2	20223044	male	horns	polycerate origin, 2			✓				✓
N°2	20223045	male	horns	polycerate origin, 2			✓				✓
N°2	20223046	male	horns	polycerate origin, 2			✓				✓
N°2	20223049	male	horns	polycerate origin, 2			✓				✓
N°2	20223058	male	horns	polycerate origin, 2			✓				✓
N°2	20223062	male	horns	polycerate origin, 2			✓				✓
N°2	20223063	male	horns	polycerate origin, 2			✓	✓			✓
N°2	20223061	unknown	horns	polycerate origin, 2			✓	✓	✓	✓	✓
N°2	20223053	female	horns	polycerate origin, 2			✓	✓			✓
N°2	20223054	female	horns	polycerate origin, 2			✓	✓			✓
N°2	20223060	male	horns	polycerate origin, 4			✓	✓	✓	✓	✓
N°2	20221862	unknown	horns	polycerate origin, 4			✓				
N°2	20211413	female	horns	polycerate origin, 6			✓		✓	✓	✓
N°2	20223051	male	horns	polycerate origin, 4			✓	✓	✓	✓	✓
N°2	20223042	female	polled	polycerate origin, 2			✓				✓

Table S3 Details on all Icelandic sheep used for the analysis. Please note that the individuals used for genotyping of the different variants are marked. (continued)

farm	**lab N°**	**sex**	**horn status**	**details on horn from or number**	**details on mothers horn phenotypes**	**details on fathers horn phenotypes**	***RXFP2* insertion (Wiedemar and Drögemüller (2015))**	**OAR10_29458450 (Dujivesteijn et al. (2018))**	**OAR10_29461968 ("Pan5", Pan et al. (2018))**	**OAR10_29462010 ("Pan6", Pan et al. (2018))**	***HOXD1* deletion (Allais-Bonnet et al. (2021))**
N°2	20223043	female	polled	polycerate origin, 2			✓				✓
N°2	20223048	female	polled	polycerate origin, 2			✓				✓
N°2	20223047	male	polled	polycerate origin, 2			✓				✓
N°2	20223055	male	polled	polycerate origin, 2			✓				✓
N°2	20223052	female	polled	polycerate origin, 2			✓	✓	✓	✓	✓
N°2	20223034	female	polled	polycerate origin, 4			✓	✓	✓	✓	✓
N°2	20223057	female	polled	polycerate origin, 4			✓	✓	✓	✓	✓
N°2	20223056	male	polled	polycerate origin, 4			✓	✓	✓	✓	✓
N°2	20223033	unknown	polled	polycerate origin, 4 (probably scurs)			✓	✓	✓	✓	✓

* no DNA samples available for analysis

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Declaration

Declaration according to the doctoral regulations of the department 09 from July 07, 2004 § 17 (2)

„Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

"I declare: I have prepared the submitted dissertation independently and without unauthorized outside help and only with the help that I have indicated in the dissertation. All text passages taken verbatim or in spirit from published writings and all information based on oral information are identified as such. In the research conducted by me and mentioned in the dissertation, I have complied with the principles of good scientific practice as laid down in the "Statutes of the Justus Liebig University Giessen for Ensuring Good Scientific Practice"."

Rebecca Simon Gießen, 12.06.2024