GENETIC BASIS FOR THE DEVELOPMENT OF SCURS IN BOS INDICUS-INFLUENCED CROSSBRED CATTLE

A Dissertation

by

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ABSTRACT

Scurs are corneous growths that develop at the same location on the skull as horns. Typically, scurs do not fuse with the frontal skull and they range from buttons to horn-like structures. Scurs are epistatic to horns and only cattle that are heterozygous at the polled locus can grow scurs. Scurs are also more frequent in males than females, suggesting sexual dimorphism. The genetic mechanisms for development of scurs and their morphology is still not clear and the mode of inheritance of scurs is debated. Therefore, the primary objective of this research was to study the mode of inheritance of scurs and better understand the genetic mechanisms behind scurs. We used heterozygous polled progeny from *Bos taurus-Bos indicus* F_2 and reciprocal backcross mapping populations. Phenotypic records and SNP genotypes were collected for cattle that were at least 18 months of age. We identified 3 types of scurs based on anatomy that can be seen as milestones in the development of horns. Two previously proposed inheritance models do not fit our populations and we found that the presence of scurs (Sc) is dominant over the absence of scurs (sc), and we suggest the presence of sheath (sh) is recessively inherited. Genome-wide association studies identified a major locus on BTA 12 that is adjacent to *RXFP2* to be associated with presence of scurs and presence of sheath. We also identified a locus on BTA 17 that is adjacent to *RXFP1* associated with presence of scurs in males, suggesting there are genetic compensation effects from *RXFP1* for development of scurs in male cattle. Spatial transcriptomics revealed scurs and horns share a similar expression profile, suggesting scurs are incomplete horns that

due to mutations affecting genes in the developmental pathway stopped at different points in development.

DEDICATION

For my mom and dad.

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Contributors

This work was supervised by a dissertation committee consisting of Professors Clare A. Gill (advisor), David G. Riley and James O. Sanders of the Department of Animal Science, Professor James J. Cai of the Department of Veterinary Integrative Biosciences.

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NOMENCLATURE

°C	degree Celsius
µl	microliter
bp	base pair
BTA	Bovine chromosome
F_1	first filial generation
F_2	second filial generation
F_2	third filial generation
GWAS	genome-wide association studies
H&E	hematoxylin and eosin
Ha	African horn allele
HD	high density
Indel	Insertion/deletion
mo	months of age
ng	nanogram
O.C.T.	optimal cutting temperature
p	horned allele
P_C	Celtic polled allele
PCR	Polymerase chain reaction
P_F	Friesian polled allele
P_G	Guarani polled allele

RNA	ribonucleic acid
Sc	presence of scurs allele
sc	absence of scurs allele
SNP	single nucleotide polymorphism
ST	spatial transcriptomics
SVs	structural variants
TADs	topologically associating domains
TIGSS	Texas A&M Institute for Genome Sciences and Society
UMAP	uniform manifold approximation and projection
UMI	unique molecule identifier
WBCs	white blood cells

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CHAPTER I

INTRODUCTION

One of the unique biological phenotypes of pecorans (even-toed ruminants) is paired and symmetric headgear (Davis et al., 2011). These osseous cranial appendages are categorized as pronghorns (in pronghorn), ossicones (in giraffids), antlers (in cervids) and horns (in bovids) (Gadow, 1902; Bubenik and Bubenik, 2012). Although the morphology of headgear is distinct in each family of pecorans, they all share a bony protuberance and a similar developmental genetic basis from neural crest stem cells (Wiener et al., 2015; Chen et al., 2019; Wang et al., 2019). Headgear is used to classify pecorans and it is seen as representative for studying the evolution of ruminants.

The horns of cattle (*Bos taurus*) have a bony core, which is covered by a non-forked, non-deciduous keratinous sheath. Development of horns begins from a separate ossification of the tissues above the frontal periosteum, which later fuses to the skull. Based on early transplantation experiments, the ossification core (or 'os cornu') lies in connective and dermal tissues. The os cornu will develop into a movable bony structure, which then fuses through the frontal periosteum to form a horn (Dove, 1935). Invasion by the frontal sinus and fusion to the skull only occur in the presence of periosteum, suggesting that multiple cell types are critical for horn formation.

Absence of horns (polledness) is a characteristic of some *Bos taurus* beef cattle breeds (e.g. Angus, Galloway) and is required for registration, so there has been strong artificial selection against the horned allele since the formation of the breeds. Indeed, in

these breeds the horned and scurred phenotypes are classified as genetic abnormalities (American Angus Association, 2022). In other breeds, there has been a more recent focus on eliminating horns because of worker safety (Stafford and Mellor, 2005) and animal welfare concerns (Wilcox et al., 2013). For example, the American British White Park Association (2020) has prohibited registration of horned animals since 2014.

Although the mechanism (i.e., molecular pathway) behind the growth of horns is still debated, genetic mutations causing polledness have been identified on bovine chromosome (BTA) 1. Polled is dominant over horns and no sex differences in the inheritance pattern have been observed. The most common polled allele (also known as the Celtic *polled* mutation, P_C) is a complex insertion/deletion (InDel) located in a non-coding region near the centromere of BTA 1. It has been reported to be a 208 bp duplication that replaced 6 bp of sequence after 10 bp of wildtype sequence or a 212 bp duplication in place of 10 bp after 6 bp of wildtype sequence (Medugorac et al., 2012; Wiedemar et al., 2014). Gene editing has confirmed that the 212 bp duplication is a functional mutation (Carlson et al., 2016). Another mutation known as the Friesian allele, P_F, is an 80 kb non-coding duplication that is about 200 kb downstream from the Celtic mutation. Two additional polled mutations, known as the Mongolian and Guarani alleles, are less well characterized and overlap with the Friesian mutation (Medugorac et al., 2012; Utsunomiya et al., 2019b). Commercial SNP arrays now include probes to test and select for both the Celtic and Friesian polled alleles (Wiedemar et al., 2014).

Since the introduction of commercial genetic tests for the polled phenotype (Gill et al., 2012), the frequency of polled animals has dramatically increased in some *Bos*

taurus beef breeds (e.g., Charolais, Limousin, and Simmental) (Beef Central, 2020). Much less progress has been made in changing the incidence of horns in *Bos indicus* beef breeds. *Bos taurus indicus* (often referred to just as *Bos indicus*) is a subspecies of cattle that originated from South Asia. *Bos indicus* breeds such as Nellore and Brahman are predominantly horned, but polled purebreds have been identified in both breeds (Stafuzza et al., 2018). The causal mutation (Guarani allele, P_G) for polled Nellore cattle is a 110 kb duplication that was mapped to the centromeric region of BTA 1, though the boundaries of the mutation have not been well characterized (Utsunomiya et al., 2019b). Based on SNP haplotypes, it has been proposed that the Guarani allele is from a taurine introgression potentially stemming from Iberian cattle imported into Brazil in 1492. Similar to Nellore, the cause of polledness in Brahman has been confirmed to be due to historic taurine introgression of the Celtic polled mutation (Koufariotis et al., 2018; Lamb et al., 2020).

In addition to horns, in cattle there is another cranial appendage called a scur. Scurs are corneous growths that range in size from buttons to large horn-like structures and develop in the same area of the frontal bone as horns, but typically do not fuse with the skull. Because of the similarity in phenotype between horns and some scurs it can be difficult to distinguish them on a live animal and misclassification has made it difficult to identify genetic loci for scurs (Davis et al., 2011).

Because scurs and horns form at the same location of the frontal bone, the development of horns will mask the status of scurs (Long and Gregory, 1978), resulting in an epistatic interaction between the polled and scurs loci. Some early observational

studies of *Bos taurus* cattle, based on population data, suggested that neither homozygous horned nor homozygous polled cattle were able to grow scurs (Lloyd-Jones and Evvard, 1916; White and Ibsen, 1936). Among *Bos taurus* breeds, it was recently confirmed that only cattle that are genotyped heterozygous polled express scurs (Wilson et al., 1974 cited by Long and Gregory, 1978; Capitan et al., 2009; Wiedemar et al., 2014). To efficiently identify the genetic basis for scurs, first-generation crossbred populations derived from homozygous polled *Bos taurus* cattle crossed with homozygous horned *Bos indicus* cattle become natural resources because every calf produced will be heterozygous polled and segregation of the scurs phenotypes can be observed. However, in most established *Bos taurus*-*Bos indicus* composites or multi-generational *Bos indicus*-influenced crossbred populations there has been segregation at the *polled* locus, and so genotyping the *polled* locus becomes important. The power of using composites (e.g., Brangus was originally intended to be approximately 3/8 Brahman and 5/8 Angus) to map the *scurs* locus will be impacted by segregation of the polled mutation. Nonetheless, while the horn (p) allele is still present at a moderate frequency in these populations, sufficient polled heterozygotes can be detected to investigate the genetic basis of the scurs phenotype. Knowledge of loci affecting absence of scurs could then be directly applied by producers to select completely against headgear (horns and scurs).

In addition to epistasis between the polled and scurs loci, the growth of scurs in males and females seems to be different, suggesting a potential sex influence (Gowen, 1918; White and Ibsen, 1936; Long and Gregory, 1978). Among heterozygous polled

cattle, males tend to grow scurs more frequently than females (Long and Gregory, 1978; Capitan et al., 2011). Scurs in females usually vary in size from scabs to large appendages, whereas scurs in males are usually large and less movable (Capitan et al., 2011). These differences are indicative of sexual dimorphism, which is very common in bovids in regard to growth of headgear (Packer, 1983; Stankowich and Caro, 2009; Johnston et al., 2011).

Unlike horns, loci controlling the presence or absence of scurs have not been definitively revealed yet. There are several studies with contradictory results that tried to investigate the inheritance pattern of scurs and map the *scurs* locus (Asai et al., 2004; Capitan et al., 2009). The first study used linkage analysis based on 162 autosomal microsatellite markers to map the *scurs* locus to BTA 19 across three full-sibling families (Asai et al., 2004). The observed phenotype was converted to a genotype for linkage analysis according to the Long and Gregory (1978) model, which proposed that one copy of the scurs allele is needed in males but two copies are needed in females for heterozygous polled cattle to grow scurs. Another study of 33 half-sibling and full-sibling French Charolais families failed to confirm the regions on BTA 19, and they also proposed there is no sex influence on scurs in French Charolais (Capitan et al., 2009). Both studies used linkage analysis to map the *scurs* locus with little information about how they scored the phenotype. Additionally, both studies were conducted before there was a bovine SNP array, which largely improved the density of markers and enabled the application of genome-wide association studies (GWAS).

One GWAS of 150 *Bos taurus* cattle heterozygous for the Celtic polled allele (P_C) found one region of weak association on BTA 19. However, they were not able to find shared homozygosity among scurred females and proposed the Long and Gregory (1978) inheritance model for scurs could be wrong (Capitan et al., 2009), and suggested there is evidence for genetic heterogeneity (Tetens et al., 2015). Another GWAS used 240 Holstein-Friesian females heterozygous for the Friesian polled allele, P_F. Similar to the first GWAS, numerous peaks were identified and they proposed that presence or absence of scurs is a quantitative trait (Gehrke et al., 2020). There were some obvious limitations in these two studies. The first study relied on a very small population and did not factor sex or the potential for different polled mutations into their model, whereas the second study focused on Holstein-Friesian cattle, all genotyped heterozygous P_F, and only females were included.

To date, the few studies of the genetics of scurs in cattle have failed to agree on the inheritance pattern of scurs leaving a major gap in knowledge of the development of these cranial appendages and limiting the ability to map and subsequently identify causative loci. The focus of this dissertation will be to understand the genetic basis of scurs in *Bos indicus*-*Bos taurus* crossbred cattle.

CHAPTER II

CATEGORIZATION OF SCURS IN *BOS INDICUS-BOS TAURUS* CROSSBRED POPULATIONS

Introduction

In cattle, the development of horns starts between 2 and 6 months of gestation. At gestational day 70, the epidermis of frontal skin is 3 layers of vacuolated keratinocytes, whereas the epidermis in horn buds is thicker and progresses from 7 to 12 layers of vacuolated keratinocytes by gestational day 115 (Konig et al., 2009; Wiener et al., 2015). The dermis is initially composed of immature collagen. By gestational day 115, thick nerve bundles form in the dermis below the horn bud but are absent from frontal skin throughout gestation. Hair follicles are present in the dermis of frontal skin by gestational day 115 and develop by day 155 in horn buds. Differentiation of sebaceous glands and epidermis occur by gestational day 155 in horn buds, which is earlier than in skin and by gestational day 212 the epidermal layer of frontal skin and horn buds cannot be distinguished.

Currently, it is agreed that ossification of horns does not occur before birth, and horn buds will remain as soft protrusions that might not be visible upon birth (Wiener et al., 2015; Li et al., 2018). From this point, the development of horns is thought to follow two parallel processes: (1) skin at the horn buds continues to keratinize into a hard integument and later forms the keratin tip and sheath of the horns; (2) a pair of primary cores form below the skin, which will later ossify and become the bony cores of horns

and fuse with the frontal skull through the frontal periosteum (Dove, 1935; Hackett et al., 2017; McGeady et al., 2017). Dove (1935) demonstrated that neither the keratinized skin can induce differentiation of the os cornu, nor can the os cornu stimulate the skin above to keratinize, suggesting the two processes were programmed separately before birth. Fusion of the bony core and frontal skull happens as early as 2 to 4 months of age, and growth of the horns can continue for as long as 4 years by which time the inside is completely pneumatized (Nasoori, 2020). Polled cattle on the other hand, do not develop any of the structures mentioned above.

The development of scurs is still debated. They are corneous outgrowths that develop at the same site as horns and generally do not fuse with the frontal skull. Although Dove (1935) argued that scurs must include bony cores, diverse morphologies of scurs have been reported since then (Blackwell and Knox, 1958; Asai et al., 2004; Capitan et al., 2009; Tetens et al., 2015; Randhawa et al., 2019; Gehrke et al., 2020). Previous studies have reported scurs as “scabs”, “buttons” or horn-like structures (Blackwell and Knox, 1958; Long and Gregory, 1978; Tetens et al., 2015; Wiener et al., 2015; Gehrke et al., 2020). Additionally, “bumps” are described in several studies, which develop on the frontal skull at the horn bud region. Bumps differ from scurs as they are not an independent development from the skull (Gehrke et al., 2020). So far, a detailed categorization of scur morphology and the corresponding frequency within a population has not been reported.

In addition to the scurs described above, on occasion there have been reports of homozygous polled bulls producing horned calves from matings with horned cows

(Long and Gregory, 1978). There is debate in the literature over whether these are firmly affixed scurs that are misclassified as horns or cases of dominant horns (Gowen, 1918; Dove, 1935; White and Ibsen, 1936). Two models developed to explain the inheritance patterns of horns specify a different independent locus such that the dominant African horn allele (*Ha*) is epistatic to the polled allele in males (White and Ibsen, 1936; Long and Gregory, 1978) to cover this type of headgear.

Instead of categorizing phenotypically different scurs separately, researchers have generally called all the morphologies above "scurs" (White and Ibsen, 1936; Long and Gregory, 1978; Capitan et al., 2009; Gehrke et al., 2020). However, it is likely that morphologically different scurs are controlled by different genetic loci and they might work chronologically. To re-investigate the development of scurs in cattle and establish a comprehensive understanding about scurs, a systematic characterization of the scurs phenotype is the first and necessary step towards this goal. Therefore, in this chapter, our objective was to categorize scurs in two *Bos indicus*-*Bos taurus* crossbred populations.

Materials and Methods

Populations

In this chapter, only previously collected animal records, photographs, and DNA samples were used. At the time the populations described herein were developed, all animal protocols were approved by the Texas A&M Agriculture Animal Care and Use Committee.

Records used in this study came from two *Bos indicus* influenced beef cattle populations: the Angleton population and the McGregor Genomics population. The Angleton population was a *Bos taurus* (Angus) x *Bos indicus* (Brahman or Nellore) double reciprocal backcross design and large full sibling families were produced by multiple ovulation and embryo transfer (Table 1). All straightbred Angus parents and grandparents were smooth polled and all straightbred Brahman or Nellore parents and grandparents were horned. Calves were born in the spring and fall from spring 1991 to spring 1996. Although the primary purpose of this population was to identify loci associated growth and carcass quality traits, the population also segregated for the polled and scurs phenotypes.

Table 1 Structure of the families from the Texas A&M University Angleton project

Family ID	Cross	Sire	Dam	Progeny
1	ABxAA[1]	U3065	X18	1
			Z6	24
2	NAxAA	2850	X18	4
			Z6	18
3	ABxAA	946	X24	0
4	NAxAA	2860	X24	1
5	ABxAA	U3065	X26	8
		P2214		8

Table 1 Continued

Family ID	Cross	Sire	Dam	Progeny
6	NAxAA	2855	X26	18
7	ABxAA	819X4	T27	33
8	NBAAxAA	58	T27	25
9	AAxAB	T5	32T	32
10	BBxAB	1\8	32T	19
11	AAxAB	Independence	X3616	11
12	BBxAB	57	X3616	19
13	AAxAB	888020	X3713	19
14	BBxAB	176	X3713	23
15	AAxAB	Y6	804/R2	21
16	BBxAB	740\7	804/R2	19
		710\6		6
17	AAxNA	T5	2853	18
18	BBxNA	1\8	2853	21
19	AAxBA	Independence	X0223	25
20	BBxBA	57	X0223	4
			P57	20
21	AAxNA	888020	2864	16
22	BBxNA	176	2864	17
23	AAxBA	Y6	X0221	9

Table 1 Continued

Family ID	Cross	Sire	Dam	Progeny
24	BBxBA	740\7	X0221	2
25	AAxBB	U3065	5\6	19
26	NAxBB	2850	5\6	17
27	ABxBB	946	613\5	1
28	NAxBB	2850	613\5	13
29	ABxBB	819X4	958\9	14
		2214		10
30	NAxBB	2855	958\9	20
31	ABxBB	819X4	748\7	3
			978\0	13
32	NBAAxBB	58	978\0	7
33	ABxAB	946	X3713	0
34	NAxAB	2850	X3713	18
35	ABxAB	U3065	804/R2	14
36	NAxAB	2850	804/R2	22
37	ABxBB	U3065	748\7	1
38	NAxBB	2850	617\5	2

[1]A – Angus, B – Brahman, N – Nellore, breed compositions were labelled following the "breed of sire, breed of dam" format.

Like the Angleton population, the McGregor Genomics population was developed to study multiple traits with a focus on lifetime productivity traits. Records used in this study were from Cycle 1 of the population, in which 13 full-sibling *Bos indicus* (Nellore) x *Bos taurus* (Angus) F_2 families were produced by multiple ovulation and embryo transfer (Table 2). Calves were born in the spring and fall from spring 2003 to spring 2007.

Table 2 Structure of the families from the Texas A&M University McGregor project

Family ID	Sire	Dam	Progeny
70	297J	431H	36
71	297J	760H	67
72	432H	511G	45
73	432H	732H	9
74	437J	640H	8
75	437J	728H	43
76	551G	664J	9
77	551G	787G	42
80	551G	429H	69
81	437J	636H	60
82	432H	559J	15
83	437J	637H	38
84	551G	911H	30

Assessment of horn status

A progressive assessment of horn status for the progeny from the Angleton population was done. Horn status was scored at birth (P for polled, H for horn buds observed), 6 months of age (1 for polled, 2, 3, 4, 5 for different sizes of scurs), and at 18 months of age (1 for polled, 2 for scurred and 3 for horned) when heifers and steers were harvested. Photographs of these cattle were taken during the growth period and, when they were harvested, photographs were taken of the frontal skull along with any horn development. Because development of scurs can be delayed compared to horns, data collected at 18 mo were used as the final horn status.

For the McGregor population, horn status was scored at 18 mo for steers when they were harvested. Like the Angleton project, photographs were taken of the frontal skull along with any headgear development. Furthermore, to better understand the anatomical differences between scurs and horns, those with a hard sheath were dissected using a band saw and photographed. For heifers that were retained for breeding, horn status was scored based on photographs taken of live animals around 18 mo.

Identification and classification of scurs

To precisely identify scurs, previously extracted DNA from all the cattle in the two populations was tested for the Celtic *polled* locus by either directly genotyping the Celtic mutation (Wiedemar et al., 2014) or indirectly by inferring the polled genotype

from the breed of origin of SNP around the Celtic mutation: cattle with two Angus haplotypes were scored homozygous polled, those with an Angus haplotype and a Brahman or Nellore haplotype were scored heterozygous polled, and those with two Brahamn or Nellore haplotypes were scored horned. Photographs collected when animals were harvested were subjected to a detailed categorization based on the anatomical structure of the headgear. Heifers that only had written records or photographs with a bad angle of the head were excluded because we were not able to precisely categorize them. Counts of the different types of scurs were summarized and compared between the two populations.

Results

Identification of different types of scurs

Records from a total of 1,086 cattle were collected from the Angleton and McGregor families shown in Table 1 and Table 2, except for 13 cattle that were not genotyped. Among these cattle, 538 cattle were genotyped to be heterozygous polled (Table 3), and of these, 468 cattle had either written phenotypic records, or photographs, or both. There were 18 cattle scored as having bumps (protrusions of the frontal skull) and each of these had 2 Angus-origin haplotypes at the *polled* locus. After checking photographs of these cattle, none of them possessed visible scurs and, most of them were visually the same as polled cattle in the photographs in terms of the appearance at the site where horns develop. Additionally, there were 6 cattle with 2 Nellore-origin haplotypes at the *polled* locus were scored as scurred. Two showed (incomplete) fusion

at the base of their scurs, two cattle showed a complete fusion with the frontal skull without much frontal sinus invasion and two were not dissected. As previously described (White and Ibsen, 1936; Long and Gregory, 1978), we also observed 23 heterozygous polled cattle that developed outgrowths like horns. The base of these scurs fused to the frontal skull and in some rare cases, the frontal sinus invaded the outgrowths. Consistent with previous reports, more scurred cattle were found among heterozygous polled steers than in heifers in both of our populations (Figure 1). Although we had more heterozygous polled cattle with phenotypic records from the Angleton population (249 cattle in Angleton vs. 219 in McGregor with phenotypic records), the McGregor population had a higher proportion of scurred cattle than Angleton (80.33% vs 62.40% regardless of sex).

Table 3 Progeny grouped by sex and genotype at the polled locus for the two populations[1]

Population	Sex	Homozygous polled	Heterozygous polled	Homozygous horned
McGregor	Male	75	134	61
	Female	47	102	50
Angleton	Male	85	155	71
	Female	70	147	76

[1] Counts include progeny with no phenotypic records for headgear status

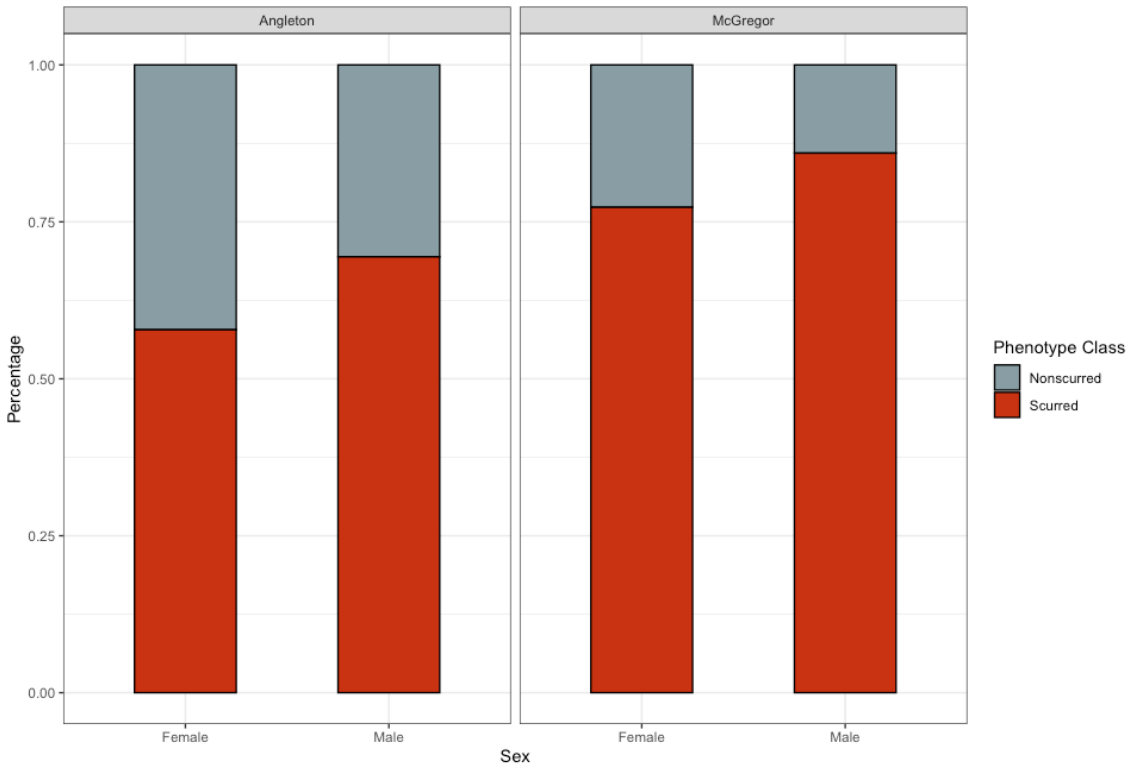

Figure 1. Proportion of nonscurred vs. scurred cattle by sex among polled heterozygotes in the Angleton and McGregor populations (n = 468)

Categorization of scurs

In both of our populations scurs had a variety of forms, as previously reported (White and Ibsen, 1936; Long and Gregory, 1978; Wiener et al., 2015; Gehrke et al., 2020). We found scurs as small as keratin patches to as large as true horns. We categorized scurs based on anatomical structures of 412 cattle with decisive photographs and showed that scurs can be classified into 3 separate types (Table 4 and Figure 2).

Table 4 Putative classification and definition of different types of scurs

Type	Definition
Type I	Thick leathery skin patch, buttons, no obvious sheath
Type II	Keratin sheath with an obvious horn-like shape, no bony core
Type III	Horn-like scurs with bony cores inside

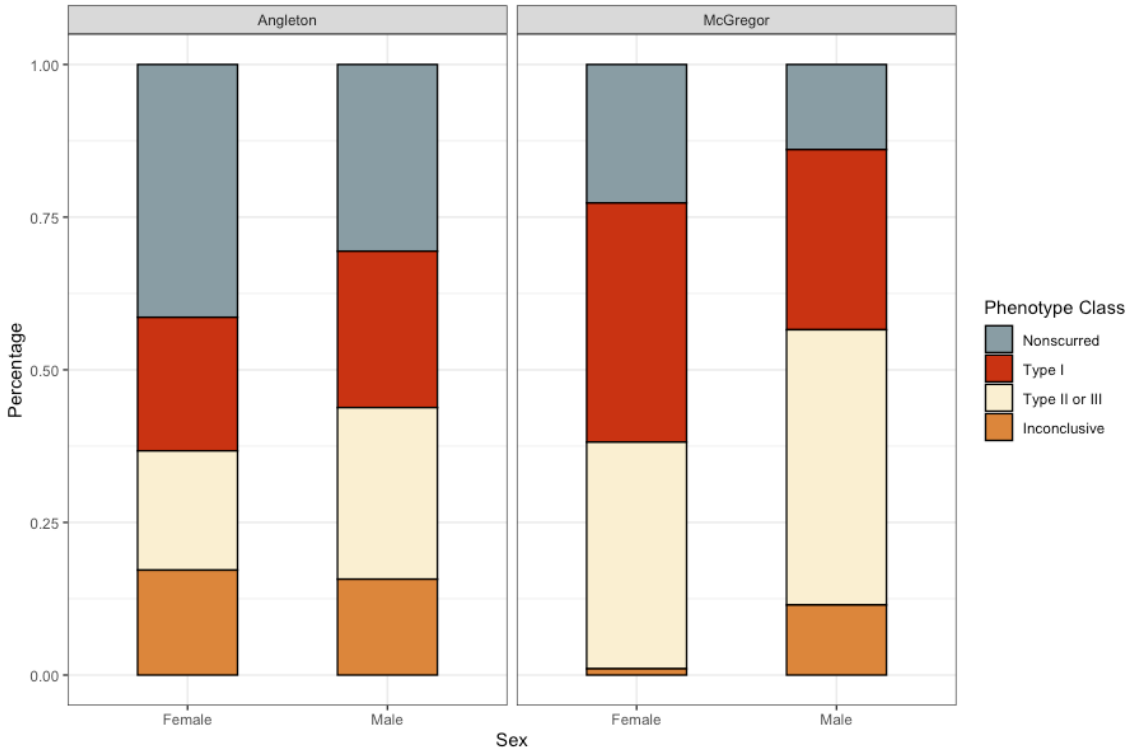

Figure 2. Proportion of different types of scurs in the Angleton and McGregor populations among polled heterozygotes (n = 468). Inconclusive (n = 56) animals were not assigned a scurs category.

Figure 3 shows a detailed annotation of each type of scurs. We expect these types of scurs are connected to how horns develop, representing three milestones in the development of horns.

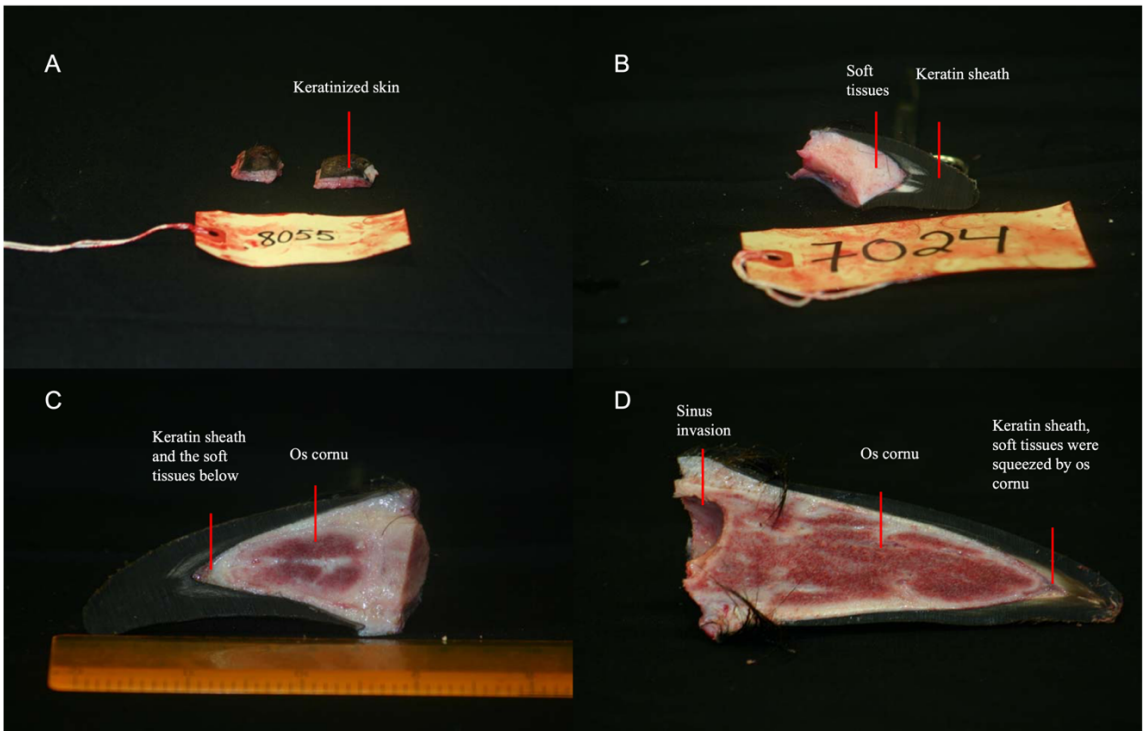

Figure 3. Structural annotation of different types of scurs and horns. (A) Type I scur. (B) Type II scur. (C) Type III scur. (D) Horn. (Gill, unpublished)

This means the later types include the features of the former types of scurs but advance to the next stage. There were also a few cases where we were not able to categorize scurs in the photographs. Failure of assignment of these scurs was usually due to them being larger than type I scurs but not having quite formed the obvious shape of type II scurs or because we do not possess a photograph of the scurs. Additionally, for the cattle from the Angleton population, there was a small proportion of scurred cattle that were scored as polled at 6 months old but were not shown to have any scurs in the photographs when they were harvested at 18 months old. These cases were included and categorized as type I scurs. Because most type I scurs have thick, keratinized skin, it is reasonable to speculate that they were hard to separate and show in the photographs. The proportion of

different types of scurs seems to be comparable between males and females, where roughly 50% of scurred males and females only developed Type I scurs (Figure 2).

CHAPTER III

REFINING THE INHERITANCE MODEL FOR SCURS

Introduction

Because scurs and horns develop in the same location on the frontal skull, the expression of scurs is epistatic to horns. The most widely discussed model for the inheritance of horns and scurs was proposed by White and Ibsen (1936) and later revised by Long and Gregory (1978). This model involves 3 biallelic loci: the *polled* locus (*P/p*), the *scurs* locus (*Sc/sc*), and the African horns locus (*Ha/ha*) (Table 5). In summary, this model proposes the following:

1. Absence of horns allele (polled, *P*) is dominant to the presence of horns allele (*p*).
2. For scurs to develop, at least one copy of the polled allele (*P*) is needed. In heterozygous polled cattle, one copy of the presence of scurs allele (*Sc*) is sufficient for males to develop scurs, whereas two copies of the *Sc* allele are needed for females to develop scurs. In homozygous polled cattle, both males and females need two copies of the *Sc* allele to develop scurs.
3. Presence of African horns allele (*Ha*) is dominant in heterozygous polled and scurred males (*PpScsc*) but recessive in females with the same genotypes at the polled and scurs loci.

A few exceptions to this model were reported in different studies. For example, Williams and Williams (1952) reported that a polled Hereford bull produced only non-

scurred progeny regardless of whether he was mated to scurred and horned cows known to transmit scurs. These observations led others to suggest partial penetrance of scurs in males (Capitan et al., 2009).

Table 5 The Long and Gregory (1978) model for the inheritance of horns and scurs

Genotype[1]	Male	Female
PPScSc	Scurred	Scurred
PPScsc	Polled	Polled
PPscsc	Polled	Polled
PpScSc	Scurred	Scurred
PpScsc	Scurred	Non-scurred
Ppscsc	Non-scurred	Non-scurred
ppScSc	Horned	Horned
ppScsc	Horned	Horned
ppscsc	Horned	Horned

[1]*P/p* – the *polled* locus; *Sc/sc* – the *scurs* locus.

Capitan et al. (2009) re-evaluated the Long and Gregory (1978) model and showed that it did not fit the inheritance patterns observed in their populations of French Charolais. Instead, they proposed a recessive model with full penetrance for the inheritance of scurs in males and females. Additionally, Gehrke (2020) proposed that the scurs phenotype is a quantitative trait because their Holstein-Friesian population showed a wide range of headgear phenotypes and they mapped 4 loci to be significantly associated with scurs, in contrast to a monogenic hypothesis.

In Chapter II, we showed the anatomical connection between scurs and horns, and that different types of scurs appear to follow the chronological development of horns. In this chapter, our objective is to test the previously proposed inheritance models for the presence of scurs and to refine the inheritance model based on categorization of scurs by morphology.

Materials and Methods

Phenotypes and pedigrees

Previously collected phenotypic records for the cattle described in Chapter II were used in this study. Based on photographs, written records and anecdotes shared by the founders of the projects (e.g., Brenneman et al., 1996), we assumed that all the F_1 *Bos taurus-Bos indicus* heterozygous polled parents in both populations were scurred. We categorized scurs in the progeny as type I, II, or III based on photographs and for those that did not have photographic records, we labelled the scur type as unknown. Because only heterozygous polled cattle can develop scurs (Capitan et al., 2009; Tetens et al., 2015; Gehrke et al., 2020), we limited the pedigrees to only show those progeny that were genotyped as heterozygous polled. Pedigree graphs were constructed using BioRender.

Inferring the genotypes

Genotypes for a biallelic scurs locus were inferred by using the phenotypic records, pedigrees, considering the assumptions of the respective models, and assuming complete penetrance. Among heterozygous polled cattle, for the Long and Gregory (1978) model, scurred females must be homozygous scurred (*ScSc*) and scurred males carry at least one copy of the Sc allele. For the Capitan et al. (2009) model, all scurred cattle must carry at least two copies of the Sc allele. For cattle whose scurs genotype could not be determined unequivocally, a likelihood calculation was carried out to determine the most likely genotype based on the pedigree. Figure 4 presents an example of a maternal half-sib pedigree that segregated for the scurs phenotype.

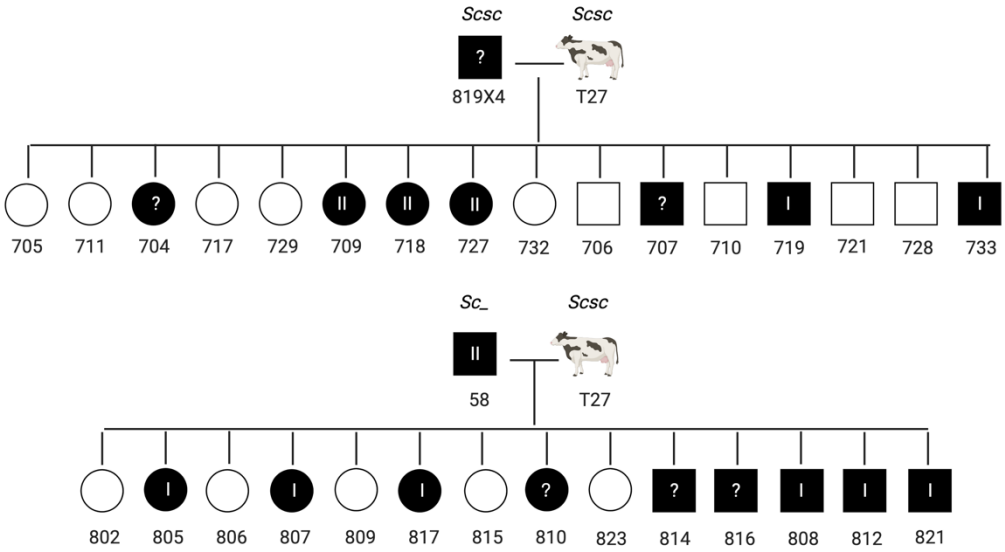

Figure 4 An example of a maternal half-sibling family. Only heterozygous polled progeny are included. Circle – female, square – male; white – nonscurred, black – scurred. I – type I scurs, II – type II scurs, ? – inconclusive.

Based on Figure 4 and assuming the Long and Gregory (1978) model is true:

1. From the family sired by 819X4 and mated to T27, it can be inferred that both 819X4 and T27 are heterozygous scurred (*Scsc*) because they produced non-scurred male and scurred female progeny.
2. For the family sired by 58 and mated to T27, the bull produced scurred female progeny which means he must carry at least one copy of the Sc allele, but the second allele could not be determined from the pedigree.
3. To calculate whether 58 is more likely to be homozygous scurred (*ScSc*) or heterozygous scurred (*Scsc*) based on the phenotypes observed in his progeny, we first labelled the probability of genotypes for every individual in this family for the two scenarios (Figure 5). For the first scenario (58 = *ScSc*) to be valid, $P(Pedigree) = 0.5^9 * 1^5 * 1$. For the second scenario (58 = *Scsc*) to be valid, we calculate the probability of the pedigree as $P(Pedigree) = 0.75^{10} * 0.25^4 * 1$.
4. Therefore, LOD can be then calculated by:

$$LOD = log_{10}(P_{58=ScSc}) - log_{10}(P_{58=Scsc})$$
$$= 0.9483$$

It is more likely that 58 is homozygous scurred based on the risk calculation, and because he did not produce any non-scurred male progeny.

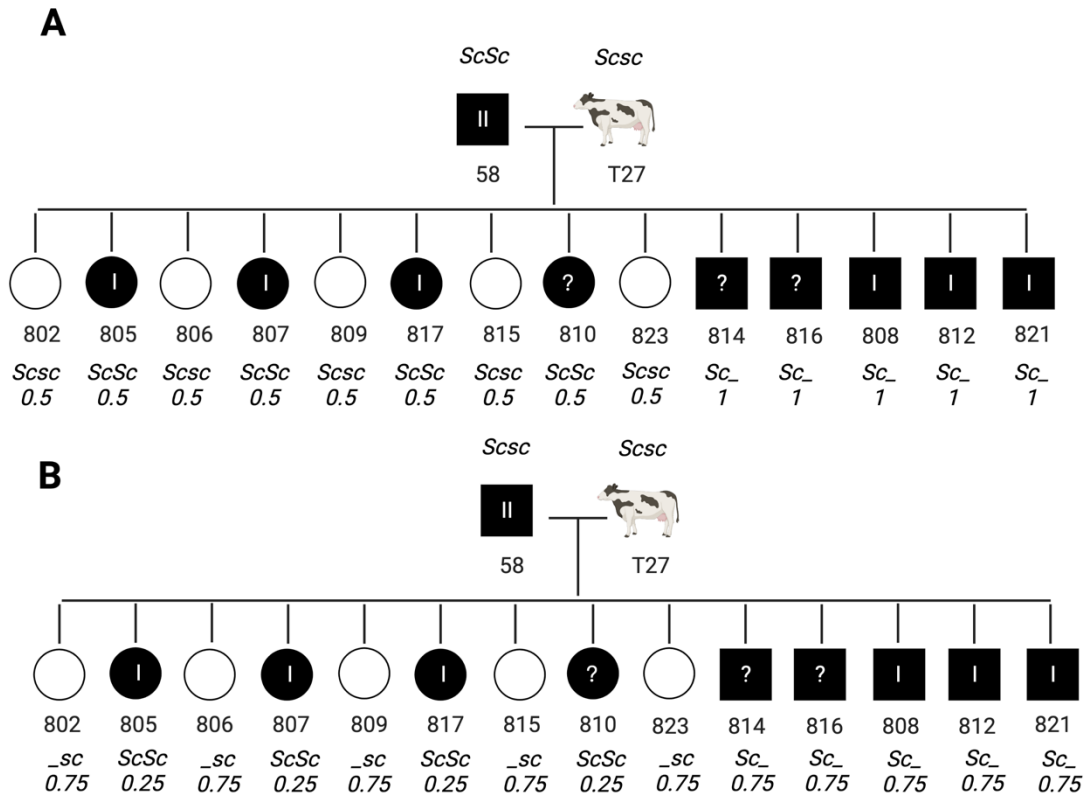

Figure 5 Genotype probability at the *scurs* locus for individuals within the family 58 x T27, assuming the Long & Gregory (1978) model is true. (A) Scenario I, 58 is assumed to be homozygous scurred. (B) Scenario II, 58 is assumed to be heterozygous scurred. Only heterozygous polled progeny are included. Circle – female, square – male; white – nonscurred, black – scurred. I – type I scurs, II – type II scurs, ? – inconclusive. *Sc* – presence of scurs allele, *sc* – absence of scurs allele, _ - either allele.

Results

Testing the inheritance models for scurs

The genotypes were inferred for the parent of each family based on the model being tested. For the Long and Gregory (1978) model, several discrepancies were observed (refer to Appendix A):

1. According to this model, scurred females must carry two copies of the Sc allele. Thus, scurred dams could not have any non-scurred male progeny. In the Angleton population, among heterozygous polled progeny, we observed such exceptions as dams 804/R2 (5 non-scurred male progeny), 32T (4 non-scurred male progeny), 2853 (6 non-scurred progeny with 5 of them produced when 2853 was mated to 1/8), X0223 (2 non-scurred progeny), X3713 (5 non-scurred progeny) and 2864 (2 non-scurred progeny).
2. Similar exceptions were found in McGregor population among heterozygous polled progeny as well. Scurred dams 760H, 511G, 551G, 728H and 636H produced a total of 12 non-scurred male progeny.

These exceptions led us to reject the Long and Gregory (1978) model in our populations. Following the same process, we tested the Capitan et al. (2009) recessive model. Numerous exceptions to the model were observed in the McGregor population:

1. Under the Capitan et al. (2009) model, in heterozygous polled cattle, the sc allele (absence of scurs) is completely dominant over the Sc allele (presence of scurs), and offspring from a scurred dam and scurred bull cannot produce any non-scurred progeny. We found contradictory results in the McGregor population as every F_2 cross was between a scurred bull and scurred dam and 12 non-scurred male progeny were produced.
2. From the family out of 32T and sired by 1/8 from the Angleton population, we can learn 1/8 must carry at least one copy of the *Sc* allele, whereas 32T is homozygous *ScSc*. However, 1/8 mated with a scurred dam 2853 produced 8

non-scurred progeny. Under this model, the probability for this pedigree is very low ($p = 0.5^8$), and they should have produced some scurred progeny.

These discrepancies led us to reject the Capitan et al. (2009) model in our populations, meaning that neither of the widely discussed inheritance models fit our populations.

Refining the inheritance model for scurs

Across the two populations, among heterozygous polled progeny, we observed a higher proportion of scurred males than females, but together they averaged 75% scurred (Figure 1). Such difference between males and females cannot be explained by the previously proposed models but may fit a dominant mode of inheritance as the frequency of scurs is close to a 3:1 ratio regardless of the sex.

Based on the results of phenotypic categorization in Chapter II, we have assumed that the scurs phenotype is controlled by several loci, and each locus represents a major developmental process such as the initiation of headgear development (i.e., presence of scurs) and the formation of structures like the keratin sheath and the bony core. However, none of the previously proposed models made clear statements about inheritance of the morphology of scurs.

Thus, to test our hypothesis that the presence of scurs allele (*Sc*) is dominant over the absence of scurs allele (*sc*), we inferred the genotypes for the parents of the two populations based on risk calculation or through pedigree information. The McGregor population is ideal for this study because all parents were scurred, whereas in the Angleton population, the scurs phenotype is always masked in the straightbred parent in

the backcross families. There were 8 McGregor families plus 2 Angleton F_2 families that were inferred as *Scsc* X *Scsc* assuming the hypothesis is true (Table 6). In this scenario, the expected ratio of scurred progeny vs. non-scurred progeny is 3:1.

Table 6 Observed and expected numbers of scurred and non-scurred progeny from 10 putative *Scsc* X *Scsc* full-sibling families and chi-square for the hypothesis that *Sc* is dominant. Genotypes for the parents of these families could be inferred unequivocally assuming a dominant inheritance model

	Observed	**Expected**
Scurred	124	121.5
Non-scurred	38	40.5
χ^2= 0.2058		

We then identified 5 full-sib families in the Angleton population that could be inferred as *Scsc* X *Scsc* and another 11 full-sib families that could be inferred as *Scsc* X *scsc* through risk calculations (Table 7 and Table 8).

Table 7 Observed and expected numbers of scurred and non-scurred progeny from 5 putative *Scsc* X *Scsc* full-sibling families in the Angleton population and chi-square for the hypothesis that *Sc* is dominant. The genotypes for the parents of these families were inferred from risk calculation assuming a dominant inheritance model

	Observed	**Expected**
Scurred	31	33.75
Non-scurred	14	11.25
χ^2= 0.8963		

Table 8 Observed and expected numbers of scurred and non-scurred progeny from 11 putative *Scsc* X *scsc* full-sibling families in the Angleton population and chi-square for the hypothesis that *Sc* is dominant. The genotypes for the parents of these families were inferred from risk calculation by assuming a dominant inheritance model

	Observed	**Expected**
Scurred	45	52
Non-scurred	59	52
χ^2= 1.8846		

As a result, based on the *Chi*-square tests for these families we are unable to reject the null hypothesis that the mode of inheritance for presence of scurs is dominant regardless of sex.

Interestingly, although the presence or absence of scurs fits a dominance model, our hypothesis is that the formation of the keratin sheath (the presence or absence of sheath, *Sh*/*sh*) is inherited as a recessive trait because we have found families in which both parents have type II scurs and more than half of their progeny also have type II scurs (e.g., McGregor family 760H X 297J and 511G X 432H). This locus is only expressed when the locus controlling the presence or absence of scurs (i.e., the *scurs* locus) is expressed, as there are non-scurred cattle produced by two scurred parents like the McGregor families mentioned above.

For the locus controlling the presence or absence of a keratin sheath, we were not able to statistically test the hypothesis for the whole population because some scurred cattle could not be categorized and performing statistical tests on the remaining cattle would introduce sample selection bias.

The mode of inheritance can be still inferred through certain families: Bull 2850 is likely *Scsc/ShSh* because he produced non-scurred calves – suggesting he is heterozygous at the *scurs* locus and most of his offspring were type II scurred when he was mated to Z6 and 613/5. When 2850 was mated to 804/R2, both type I scurred and type II scurred progeny were produced. This suggests that 804/R2 is heterozygous *Shsh*, which according to the hypothesis, cannot grow a keratin sheath and based on photographs, she had type I scurs.

CHAPTER IV

GENOME-WIDE ASSOCIATION STUDY FOR SCURS IN NELLORE-ANGUS CROSSBRED CATTLE AND BRAHMAN-ANGUS RECIPROCAL BACKCROSS CATTLE

Introduction

Although the mode of inheritance of scurs has been studied previously (White and Ibsen, 1936; Long and Gregory, 1978; Capitan et al., 2011; Gehrke et al., 2020), mapping of the scurs locus has been hindered by several challenges. The main reason is the epistatic effect of the *polled* locus over the *scurs* locus. Because horns and scurs develop in the same area, the presence of horns masks development of scurs. In addition, as we showed in Chapter II and others have shown previously (Gehrke et al., 2020; Ketel and Asai-Coakwell, 2020), homozygous polled cattle do not seem to be able to grow scurs, leaving only heterozygous polled cattle that are able to express scurs. Consequently, developing large populations with sufficient power to map the scurs locus has been difficult.

Another factor is the diverse morphology of scurs. Previous research considered the morphology of scurs to be controlled by other loci (Gehrke et al., 2020), but the presence or absence of scurs is due to the *scurs* locus. As we showed in Chapter II, different types of scurs are physiologically different, and this suggests that more than one gene could be involved in the development of the different morphologies. In earlier

studies, all types of scurs, regardless of underlying anatomy, were considered the same phenotype.

Finally, sexual dimorphism, both in terms of morphology and the frequency of occurrence of scurs, has been described previously and observed in our populations. More scurs were observed in males than in females in most previous studies (White and Ibsen, 1936; Blackwell and Knox, 1958; Long and Gregory, 1978) and in our research, and the mode of inheritance for scurs may differ in males and females. Furthermore, scurs were found to be generally larger in males than in females (Asai et al., 2004), suggesting possible hormonal regulation of the development of scurs (Blackwell and Knox, 1958). Though the reason for the sex influence is still debated, sex is a factor to consider when trying to identify the genetic mechanisms for scurs.

There were several previous studies that attempted to map the scurs locus. Asai et al. (2004) mapped the scurs locus in three beef cattle families using 162 microsatellites, and they found an associated locus on bovine chromosome (BTA) 19. They assumed the Long and Gregory (1978) model was true and only heterozygous polled cattle were included. Cattle were coded as polled or scurred without taking sex into consideration. Capitan et al. (2009) evaluated the Long and Gregory (1978) model and tried to validate the assignment of scurs to BTA 19 (Asai et al. 2004) by performing linkage analyses using a few markers within the 14.8-cM interval of BTA19 and failed to reproduce a signal on BTA19. They also proposed that the mode of inheritance of scurs is recessive in French Charolais (Capitan et al., 2009). Additionally, Gehrke et al.

(2020) mapped several loci across the genome and proposed that scurs is a quantitative trait.

However, there are limitations in each of these previous studies. The results in Asai et al. (2004) were based on the assumption that the mode of inheritance proposed by Long and Gregory (1978) was correct, even though no studies have validated the model. Neither Capitan et al. (2009) nor Gehrke et al. (2020) accounted for sex differences in their analyses. Moreover, the morphology of scurs was ignored in the mapping analysis.

The objective of this chapter is to map loci associated with three categories of scurs using genome-wide association studies.

Materials and Methods

Animals

All animal protocols used in this study were approved by the Texas A&M Agriculture Animal Care and Use Committee (AACUC).

Records for cattle described in Chapter II (Table 1 and Table 2) were used in this study. Moreover, for the purpose of mapping the presence or absence of scurs, an additional 164 McGregor Cycle 2 and Cycle 3 cows were included in the analysis by scoring the headgear on live animals. Cycle 2 cows were from natural service half sibling families representing all four reciprocal F_2 crosses between Angus (A) and Nellore (N): AN x AN, AN x NA, NA x AN, NA x NA. Cycles 3 cows are the F_3 generation out of Cycle 1 cows and by Cycle 1 bulls.

Blood sample processing and DNA isolation

Previously extracted DNA samples were available for most of the Angleton and McGregor populations (Brenneman et al., 1996; Hulsman Hanna et al., 2014). Because the extracted DNA had been at -80°C for at least a decade, we checked the integrity of the DNA by electrophoresis on a 1% agarose gel and determined DNA concentration using a Nanodrop. Degraded samples were re-extracted from stored white blood cells (WBCs) of the same cattle.

For the additional McGregor Cycle 2 and Cycle 3 cattle used in this chapter, blood samples were received between 2006 and 2014 and WBCs were isolated using sucrose-triton and stored at -80°C. DNA was extracted for these samples in 2018 for a longevity study (Engle et al., 2018). Briefly, DNA was extracted from the WBCs by proteinase K digestion and purified by using a 25:24:1 phenol:chloroform:isoamyl alcohol method. Concentration of DNA was determined by using a Nanodrop and the integrity was checked by electrophoresis of 1 µl DNA samples on 1% agarose gels in 1x Tris-acetate-EDTA buffer. All samples were normalized to a working DNA concentration of 50 ng/µl.

Genotyping and sequencing

Genotypes for McGregor Cycle 1 cattle had been previously obtained for other studies using the Illumina BovineSNP50v1 or BovineSNP50v2 chip (Illumina, San Diego, CA). For the other animals, diluted DNA samples in 96-well format were shipped to Weatherbys (Ireland) for SNP genotyping. The Weatherbys Bovine VersaSNP 50K has 42,606 SNP in common with the Illumina BovineSNP50 chip and it

has other customized markers designed for most economic traits, including the Celtic *polled* locus. High density (770K) genotypes using the Illumina BovineSNPHD Chip (Illumina, San Diego, CA) were obtained for the parents and grandparents that contributed to at least 10 progeny in the Angleton and McGregor populations.

For the 13 founders of the McGregor population including 7 Nellore (*Bos taurus indicus*) and 6 Angus *(Bos taurus taurus*) cattle, high-quality DNA of these cattle was previously sent to Illumina for library construction 100 bp paired-end sequencing using a HiSeq2000. Each sample was sequenced to ~30X depth of genome coverage, returning ~80 Gb reads.

Processing data

The SNP manifests for the Illumina 50K and HD chips use coordinates from the UMD 3.1.1 reference assembly (Bovine Genome Sequencing and Analysis Consortium, 2009; Zimin et al., 2009). With the release of ARS-UCD1.2 bovine reference assembly (Rosen et al., 2020) that utilized PacBio sequencing to construct a long-read based reference genome with fewer gaps in the genome, we obtained the probe sequences for all SNP markers from the Illumina 50K and HD chips and mapped them to ARS-UCD1.2 using Blast (Altschul et al., 1990) to lift over the SNP coordinates to the new assembly. Probe sequences for SNP markers that did not have a unique match in ARS-UCD1.2, or if their coordinates were largely different (coordinates mismatch > 2 bp) than coordinates obtained by Schnabel (2019) were excluded from all downstream analysis. Slightly different mappings for SNP markers (coordinates mismatch <= 2 bp) were retained, and the liftover-coordinates were kept because long-read-based PacBio

reference genome performs better in closing genomic gaps. Summary statistics of the lift over results compared to the existing lift over to ARS-UCD1.2 can be found in Appendix B.

Both 50K and HD genotypes were filtered using Plink v2.1 (Chang et al., 2015) to retain individuals with no more than 5% missing genotyping rate, retain SNP with minor allele frequency higher than 10% and to remove SNP deviating from Hardy-Weinberg equilibrium proportions at $P < 0.0001$, for all cattle regardless of the availability of phenotypic records.

Genomic relationship matrices of each chromosome were calculated for all cattle from the combined population using Gemma (Zhou and Stephens, 2012) while applying the Leave-One-Chromosome-Out (LOCO) method (Sul et al., 2018). The LOCO method controls the bias being introduced by testing the SNP twice in both the kinship matrix and association. All cattle with or without phenotypic records were included and a standard relatedness matrix (Zhou and Stephens, 2012) was calculated using the following equation, where X is a $n \times p$ matrix of genotypes, X_i is the ith SNP genotype and v_{x_i} is the sample variance of the ith SNP.

$$G_s = \frac{1}{p}\sum_{i=1}^{p}\frac{1}{v_{x_i}}(\mathrm{X}_i - 1_n\bar{x}_i)(\mathrm{X}_i - 1_n\bar{x}_i)^T$$

Genome-wide association studies

All genome-wide association studies were carried out using Gemma (Zhou and Stephens, 2012). To better dissect the genetic basis for scurs, we used different phenotypic coding systems to map the genetic factors behind them:

1) presence of scurs (Table 9). All cattle were heterozygous polled. The 468 cattle shown in Figure 1 from Chapter II and an additional 164 cattle from McGregor Cycle 2 and Cycle 3 were used.

Table 9 Coding system for GEMMA to map the presence of scurs locus

Type	Description	Coding in GEMMA
Non-scurred	Smoothly polled	0
Type I scur	Thickened skin, keratin patch, no hair observed, no ossification	1
Type II scur	Sheath formed with a clear shape, no ossification	1
Type III scur	Type II scur with an ossification center	1
Others	No record, unable to characterize due to blurred photographs, etc.	-1

2) type 1 scurs vs. all other types (Table 10). All cattle were heterozygous polled. For this analysis, only cattle for which the morphology of scurs could be decisively determined were included (N = 412). Cows from McGregor Cycle 2 and Cycle 3 (N = 164) were excluded as they only had written records.

Table 10 Coding system for GEMMA to map the locus associated with the development of a scur with a keratin sheath

Type	Description	Coding in GEMMA
Non-scurred	Smoothly polled	0 or -1*
Type I scur	Thickened skin, keratin patch, no hair observed, no ossification	0
Type II scur	Sheath formed with a clear shape, no ossification	1
Type III scur	Type II scur with an ossification center	1
Others	No record, unable to characterize due to blurred photographs et al.	-1

Gemma applies a univariate linear mixed model and cattle assigned as Others in each coding system (i.e., no phenotypic records or no detailed characterization of scurs due to no dissection information), were coded as "-1" to exclude them from the association tests. The significance threshold ($p = 0.05$) was adjusted using the FDR approach (Kaler and Purcell, 2019).

Fine mapping of the scurs locus

To better refine the *scurs* locus, we used HD genotypes from the parents and grandparents to impute the 50K genotypes of offspring to HD density. To achieve this, we recreated the two-step method from Rowan et al. (2019) using our own multi-population reference panel. We first combined the HD genotypes of the parents and grandparents from the Angleton and McGregor populations to generate a reference panel. Plink v2.1 and bcftools v1.9 (Li, 2011) were used to remove potential duplicate

SNP markers as the HD genotypes were provided by two different companies and some markers had different names but the same genomic coordinates.

Analysis of haplotype and breed-of-origin effects

To better investigate the mode of inheritance of scurs, we constructed haplotypes around the peak signals obtained from GWAS for the whole population. fastPHASE (Scheet and Stephens, 2006) was used to phase a 1.5-Mb window surrounding the peak signals. Haplotypes were later re-organized according to their parental origin and we tracked the flow of haplotypes within each family.

Because the growth of headgear in cattle exhibits strong breed-of-origin effects, we also assigned the breed-of-origin to each haplotype based on the haplotypes of the straightbred founders and assuming there was no introgression from another breed in the regions of interest. The breed-of-origin effects on phenotypic distributions as well as transmission of this region were manually investigated through each family.

Identification and assessment of putative causative mutations for scurs

The sequences of the founders of the McGregor population were mapped to the ARS-UCD1.2 cattle reference genome using bwa-mem (Li and Durbin, 2010) with default parameters. The GATK pipeline (DePristo et al., 2011) was then used to identify SNP and structural variants (SVs).

We manually walked through the *polled* locus as well as the regions identified in GWAS to investigate large SVs as potential causal candidates for scurs. For these identified SVs, we designed primers using the flanking regions and set up a PCR panel

to test the association between the SV and phenotypic variations. Because the founders are either straightbred Angus or Nellore, we also assigned breed-of-origin to SVs based on which founder possessed the SV. We also confirmed the assignment of breed-of-origin to the variants by mapping the regions containing SVs to Brahman and Angus haplotype-based reference sequences [GCF_003369695.1]), provided by University of Queensland.

To assess the potential effects of identified SVs, we recruited machine learning to computationally evaluate mutations. The effects of mutations on chromatin re-arrangements were evaluated by DeepC (Schwessinger et al., 2020), a machine-learning-based method to predict genomic re-arrangements based on DNA sequence. This method utilized real chromatin data captured by High-throughput Chromosome Conformation Capture (Hi-C) technique to obtain a trained model first, then predictions were made by submitting DNA sequence to the trained model using TensorFlow (v1.7) (Paramasivam et al., 2020). Because we do not have real chromatin data from cattle, we used a model trained on human Hi-C data to predict the genomic arrangements. Cross-species predictions have been previously tested by the authors of DeepC using human and mouse DNA sequences (Schwessinger et al., 2020).

Results

Genome-wide association studies for the scurs locus

Our objective was to map the *scurs* locus using GWAS and our hypothesis was that the *scurs* locus controls the presence or absence of scurs in heterozygous polled cattle. Because scurs show strong sexual dimorphism in terms of the frequency of scurred cattle and previous studies have suggested a different mode of inheritance of scurs in males and females, we performed GWAS for males and females independently using the same phenotypic coding system (Table 3).

There were 243 males (55 nonscurred, 188 scurred) and 389 females (111 nonscurred, 278 scurred) used for GWAS. We identified one significant position on BTA17 for males (Figure 6) and a strongly associated region on BTA12 for females (Figure 7). We also identified 4 other significantly associated markers on BTA7, 18, 23 and 29 for females.

As we described in Chapter II, the different morphologies of scurs appear to be due to sequential stopping points in the development of horns and each type of scurs possesses something new compared with the previous type. Thus, to find associated loci for the different types of scurs we recoded our phenotypic system in GEMMA. To test for loci associated with the development of the sheath (type II scur) rather than a keratin patch (type I scur), we recoded the phenotypes as shown in Table 10. The challenge here is whether non-scurred cattle should be included in this test. To find out if there are genetically fundamental differences between non-scurred cattle and type I scurred cattle,

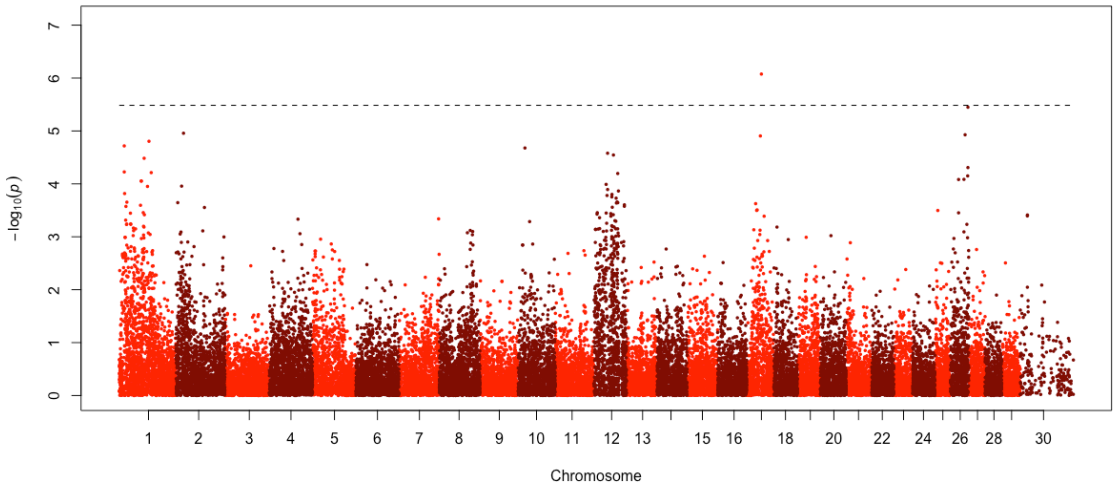

Figure 6 Genome-wide associations for the presence or absence of scurs for males (n = 243). Dotted line represents the FDR-adjusted threshold p-value = 0.05.

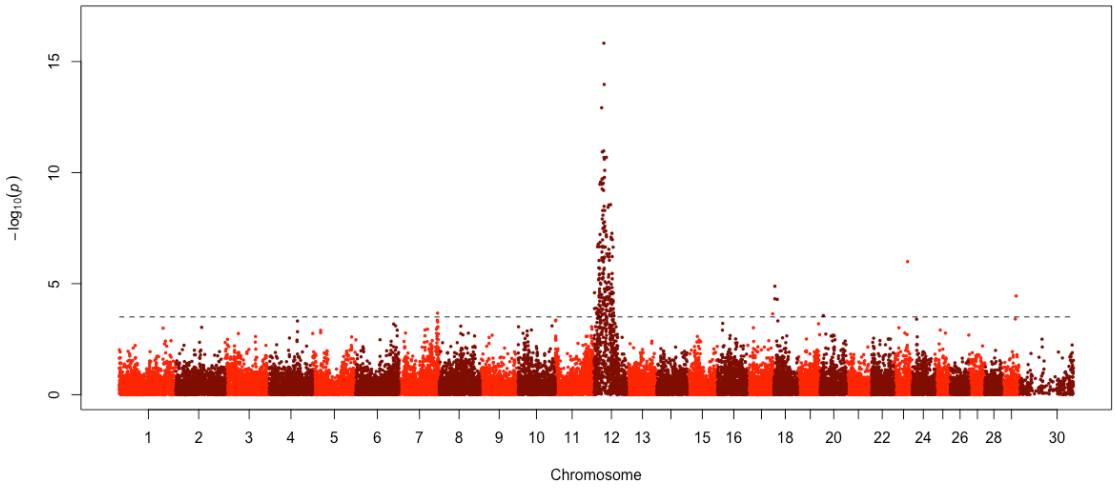

Figure 7 Genome-wide associations for the presence of scurs for females (n = 389). Dotted line represents the FDR-adjusted threshold p-value = 0.05.

we created two systems with one of them including the non-scurred cattle and coded them the same as type I scurs, and in the other system non-scurred cattle were omitted. The two systems can be used to help us understand the genetic differences between non-scurred cattle and type I scurred cattle – do non-scurred cattle and type I scurred cattle share the same genetic variants that differ in type II or III scurred cattle?

Again, separate GWAS were performed for males and females. Omitting the non-scurred cattle, a total of 155 males and 127 females were included in the GWAS. For males, we mapped two major loci associated with the development of a scur with a keratin sheath, with one of the loci being the same locus we mapped for the presence or absence of scurs in females (Figure 8). For females, there were no SNP associations that passed the adjusted *p*-value threshold (Figure 9).

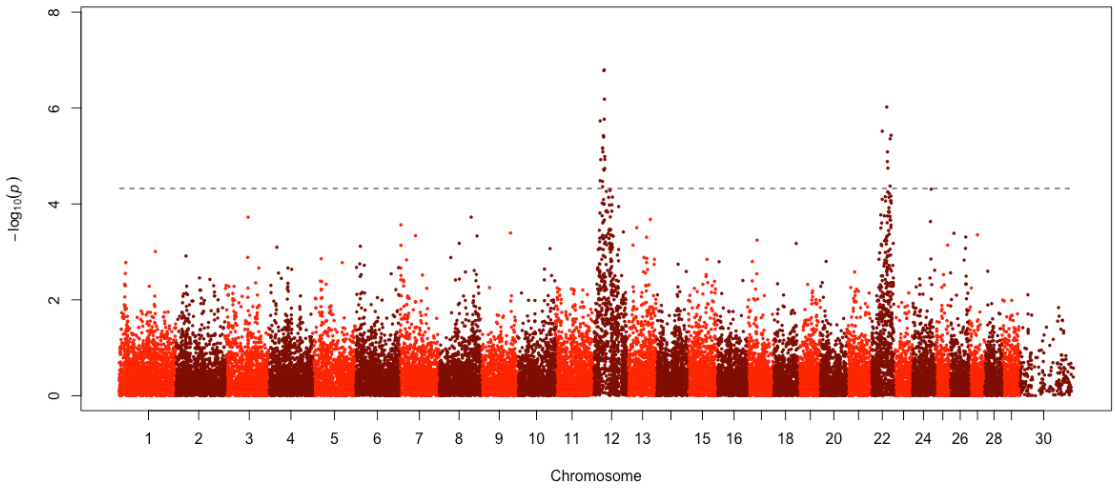

Figure 8 Genome-wide associations for the development of scurs with a keratin sheath in males (n = 155). All cattle were genotyped as heterozygous polled and nonscurred cattle were excluded. Dotted line represents the FDR-adjusted threshold p-value = 0.05.

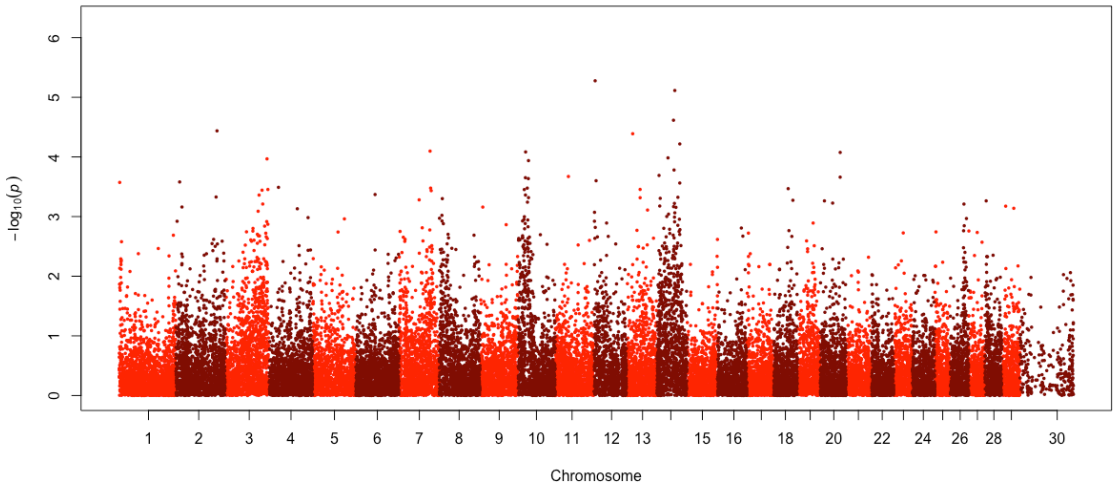

Figure 9 Genome-wide associations for the development of scurs with a keratin sheath in females (n = 127). All cattle were genotyped as heterozygous polled and nonscurred cattle were excluded. Dotted line represents the FDR-adjusted threshold p-value = 0.05.

Once we included the non-scurred cattle, an additional of 55 males and 74 females were included in the analyses. Adding non-scurred male cattle increased the significance of the associations we mapped, and the associations on BTA 12 were detected again in females (Figures 10 and 11).

These results indirectly suggest that the development of type I scurs may be due to a different locus at least in males. We obtained a stronger association at the same locus on BTA12 with non-scurred cattle added, which suggests that non-scurred cattle share the same genetic variant that differs between type I scurs and type II scurs.

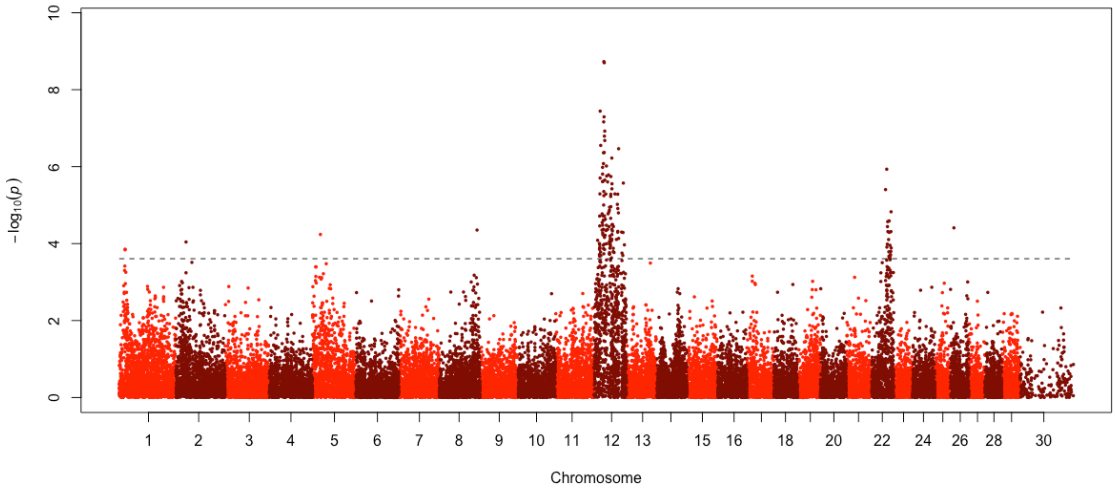

Figure 10 Genome-wide associations for the development of scurs with a keratin sheath in males. All cattle were genotyped as heterozygous polled. In this comparison, an additional of 55 nonscurred cattle were included (n = 210). Dotted line represents the FDR-adjusted threshold p-value = 0.05.

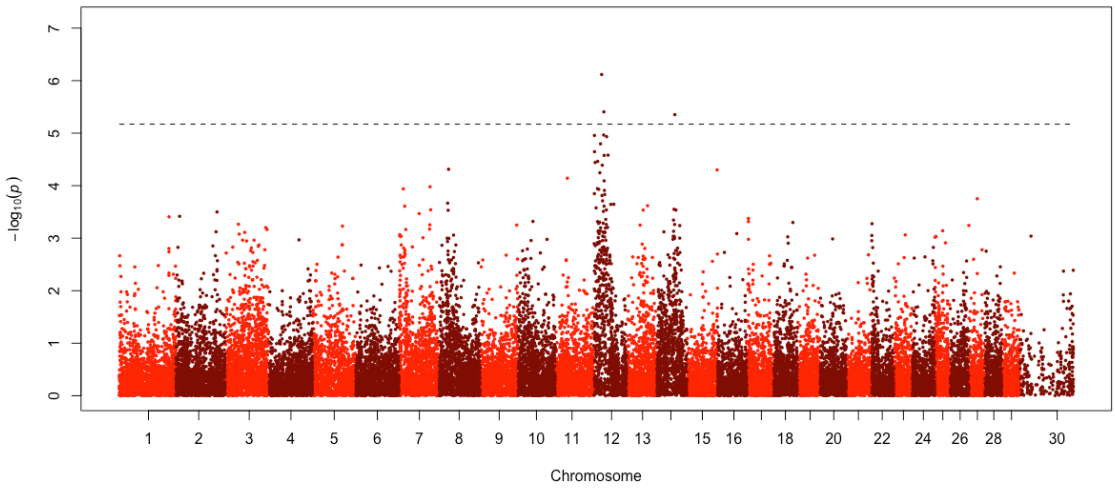

Figure 11 Genome-wide associations for the development of scurs with a keratin sheath in females. All cattle are heterozygous polled. In this comparison, an additional of 76 nonscurred cattle were included (n = 203). Dotted line represents the FDR-adjusted threshold p-value = 0.05.

Our next step was to carry out GWAS to map loci for the presence or absence of bony cores, but no significant locus was identified, most likely due to the lack of power because we were not able to identify all the bony scurs without photographs for the dissected scurs.

The peak on BTA12 was observed in several of the GWAS comparisons, suggesting its strong association with the scurs and sheath phenotypes. Within 2 Mb flanking the lead SNP markers on BTA12 (between Hapmap25509-BTA-126632 at 28,275,938 bp and ARS-BFGL-NGS-76809 at 29,238,096 bp), genes such as *PDS5B*, *N4BP2L2*, *N4BP2L1*, *BRCA2*, *ZAR1L*, *FRY*, *RXFP2*, *B3GLCT*, *HSPH1* are included (Figure 12). Among these genes, Relaxin Family Peptide Receptor 2 (*RXFP2*) is one of the most studied genes associated with headgear formation in other species (Johnston et al., 2013; Kardos et al., 2015; Wang et al., 2019). We also investigated the region containing the lead SNP marker on BTA17 (ARS-BFGL-NGS-102428 at 40,643,707 bp), which was associated with the presence or absence of scurs for males, and this region contains the Relaxin Family Peptide Receptor 1 (*RXFP1*). The region mapped on chromosome 22 contains genes such as *ASB14*, *APPL1*, *HESX1*. Among these genes, *APPL1* (coding for adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1) mediates adiponectin signaling to inhibit osteoclastogenesis and bone resorption (Tu et al., 2011).

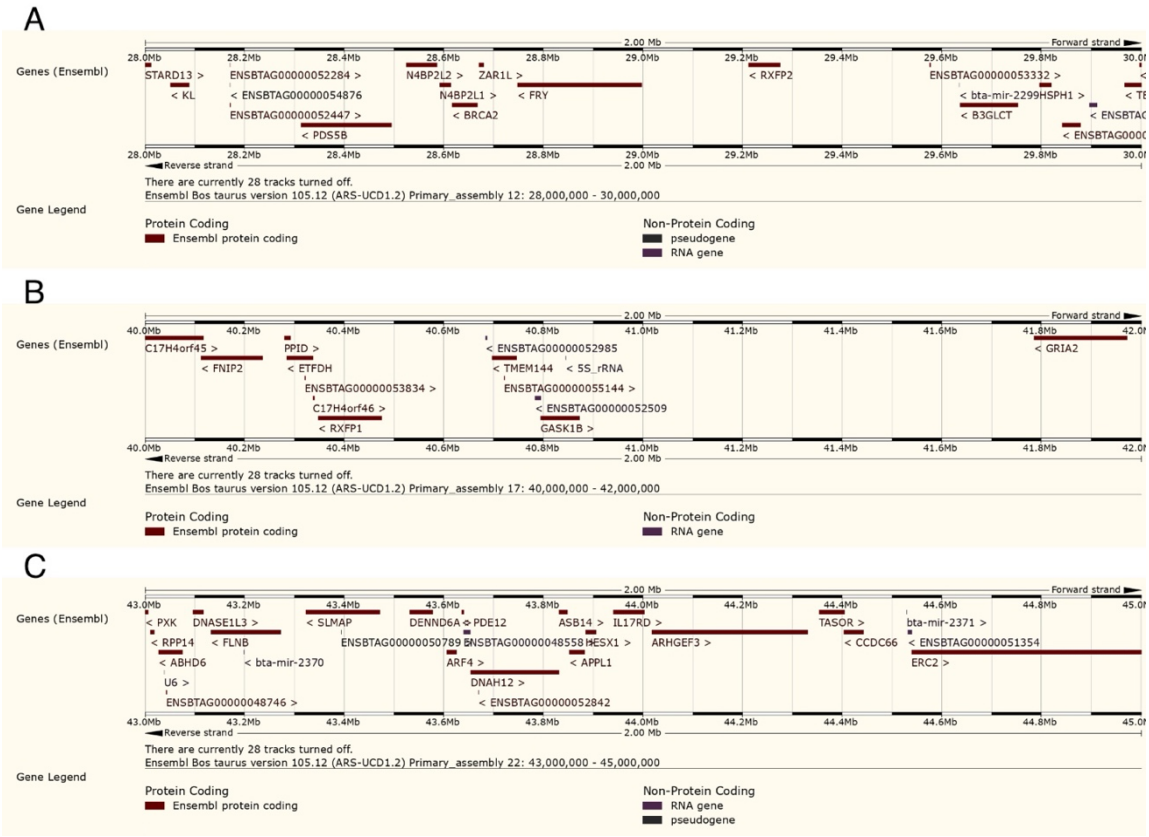

Figure 12 Annotated GWAS peak region (2 Mb region centered on the peak SNP marker) on (A) BTA12; (B) BTA17; (C) BTA22. Annotations are from Ensembl and only protein-coding genes (both predicted and validated) were included.

Haplotype analysis of the mapped regions

To better understand the inheritance of scurs based on the regions we mapped in males and females, we studied haplotype transmission within each family of the two populations. We first looked at the overall haplotype frequency within the regions of interest. Phased haplotypes were supplied to generate clusters of haplotypes and estimate the frequency of each cluster. Because the number of clusters (K) needs to be carefully determined, several scenarios were tested, such as K=2 (*Bos taurus taurus* vs. *Bos taurus*

indicus), K=3 (Angus, Brahman, Nellore) or K=15 (determined by software algorithm). As a result, we observed that the frequency of certain haplotypes increased in scurred cattle at the *RXFP2* region, regardless of the selection of K (Figure 13 and Figure 14). This confirms that there are substantial changes in terms of allele frequency associated with the phenotype.

To understand the association of scurs with the regions of interest, we phased a 1-Mb region containing 11 markers centered around the lead SNP (BTA12:28275938) and compared the transmission of haplotypes with the distribution of phenotypes. As a result, we found that the transmission of haplotypes at *RXFP2* region alone cannot explain all the phenotypic variation we see in the populations, meaning there are cattle within the same family possessing the same combination of haplotypes that express different phenotypes (Figure 15). The conflicts between haplotype transmission and phenotypic differences suggest that potentially more than one locus is involved in this developmental process.

We next tried to evaluate if there are any potential breed-of-origin effects, since breeds such as Angus were intensively selected for the polled phenotype whereas there was no artificial selection against horns in breeds like Nellore and Brahman. Based on the breed of the founders in both populations, we manually assigned haplotypes to each breed by tracing the haplotypes in the progeny back to the founders (i.e., *Bos taurus*-origin or *Bos indicus*-origin) for each family. Combined with the categorization of scurs, we found that cattle possessing two *Bos taurus*-origin haplotypes may not be able to

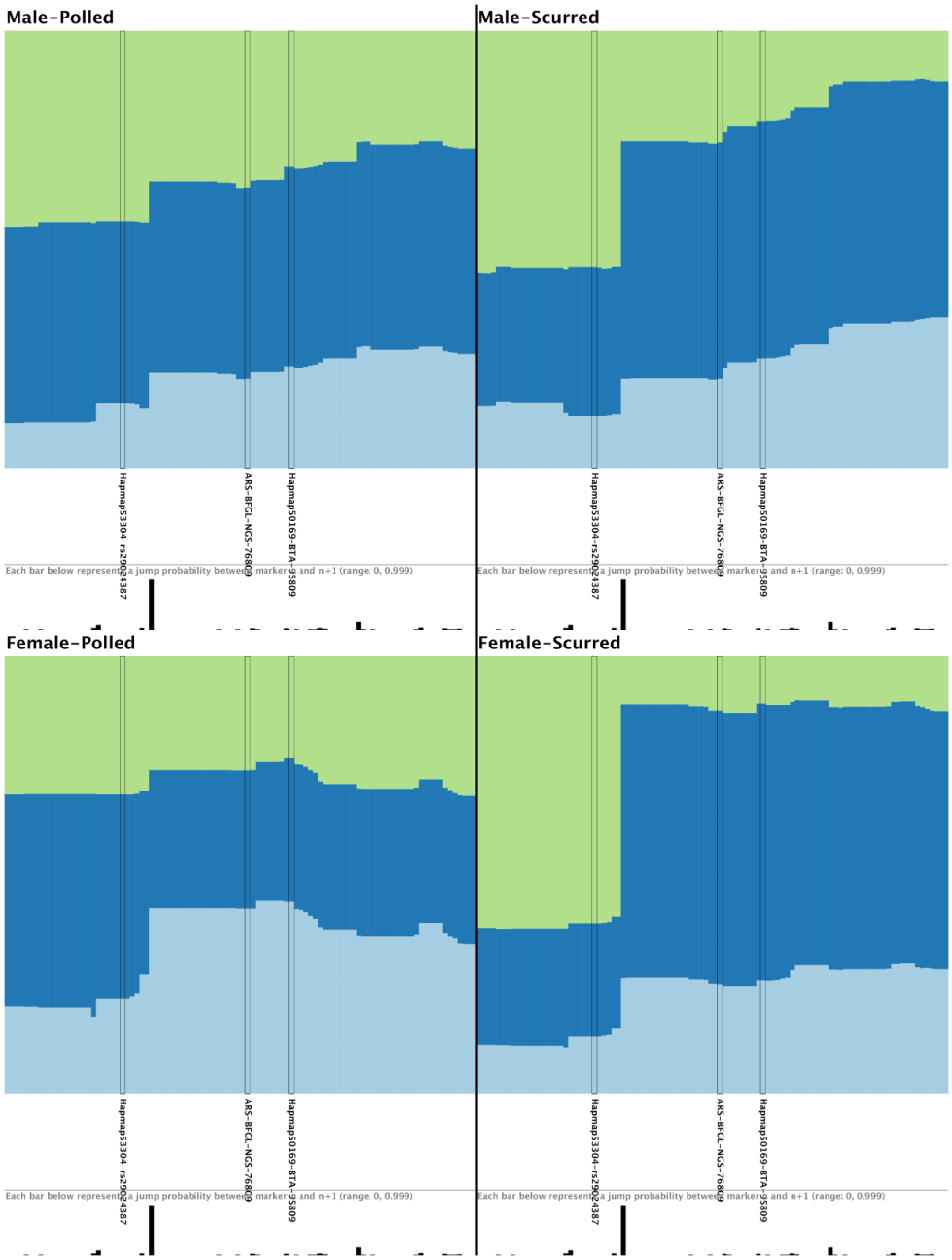

Figure 13 Clusters of haplotypes in the *RXFP2* region with K = 3. Three markers around the gene body of *RXFP2* are labeled. Each color represents a statistically clustered haplotype. K was chosen based on there being three different breeds (Angus, Nellore and Brahman) in the populations.

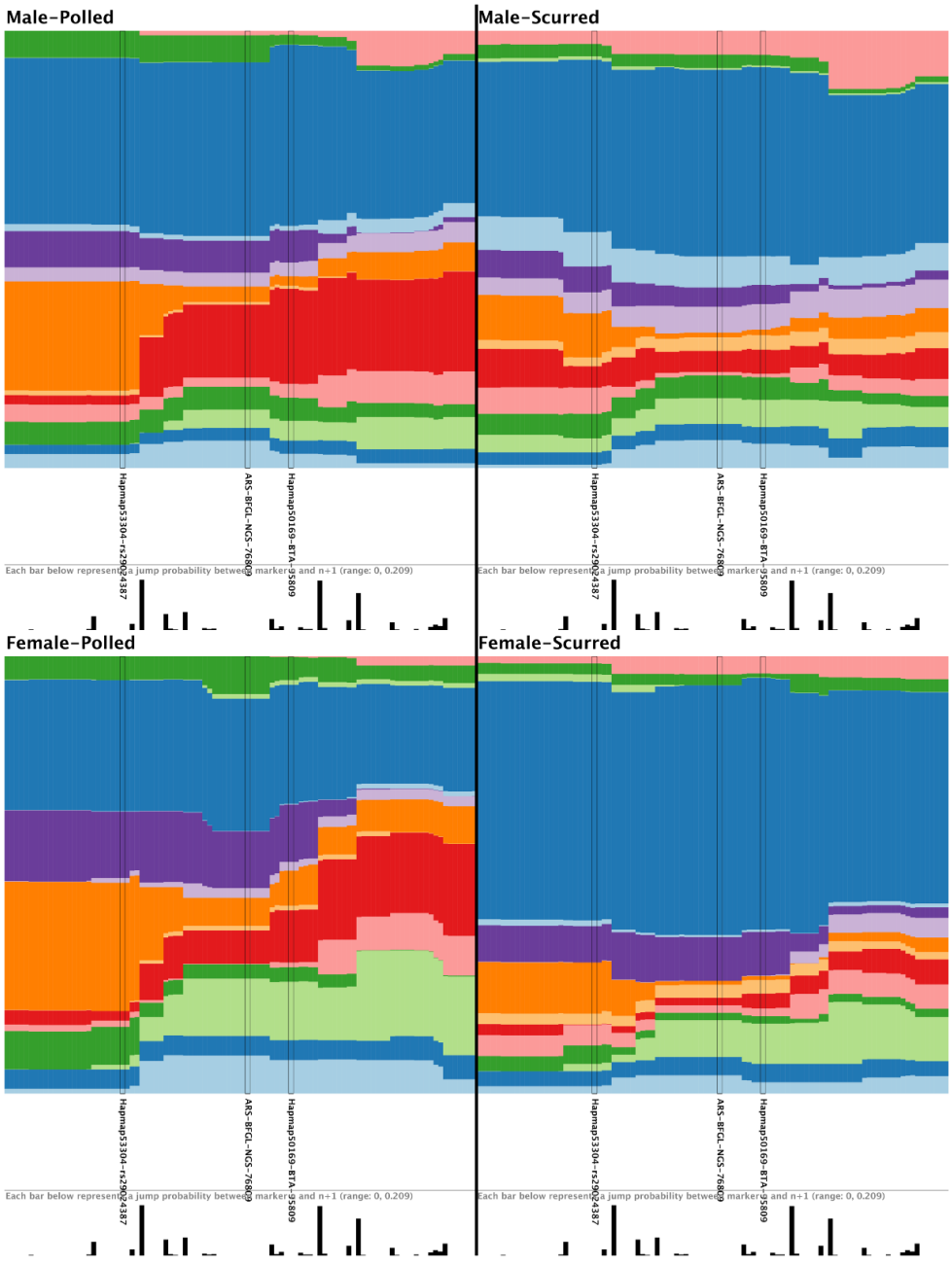

Figure 14 Clusters of haplotypes at *RXFP2* region with K = 15. Three markers around the gene body of *RXFP2* are labeled. Each color represents a statistically clustered haplotype. K was determined by Haploscope.

grow scurs beyond type I scurs (thickened skin/keratin patch) with only 2 exceptions among males from family 1 of Angleton population (Figure 15). The possibility of the 2 exceptions being mistakenly labelled or collected cannot be ruled out. More developed scurs (type II and type III scurs) were observed in cattle with 2 copies of *Bos indicus*-origin haplotypes than cattle with 1 copy of *Bos indicus*-origin haplotype. This result indicates that the variants found in *Bos indicus* breeds at *RXFP2* region play a role in both the frequency of scurred cattle and size of scurs.

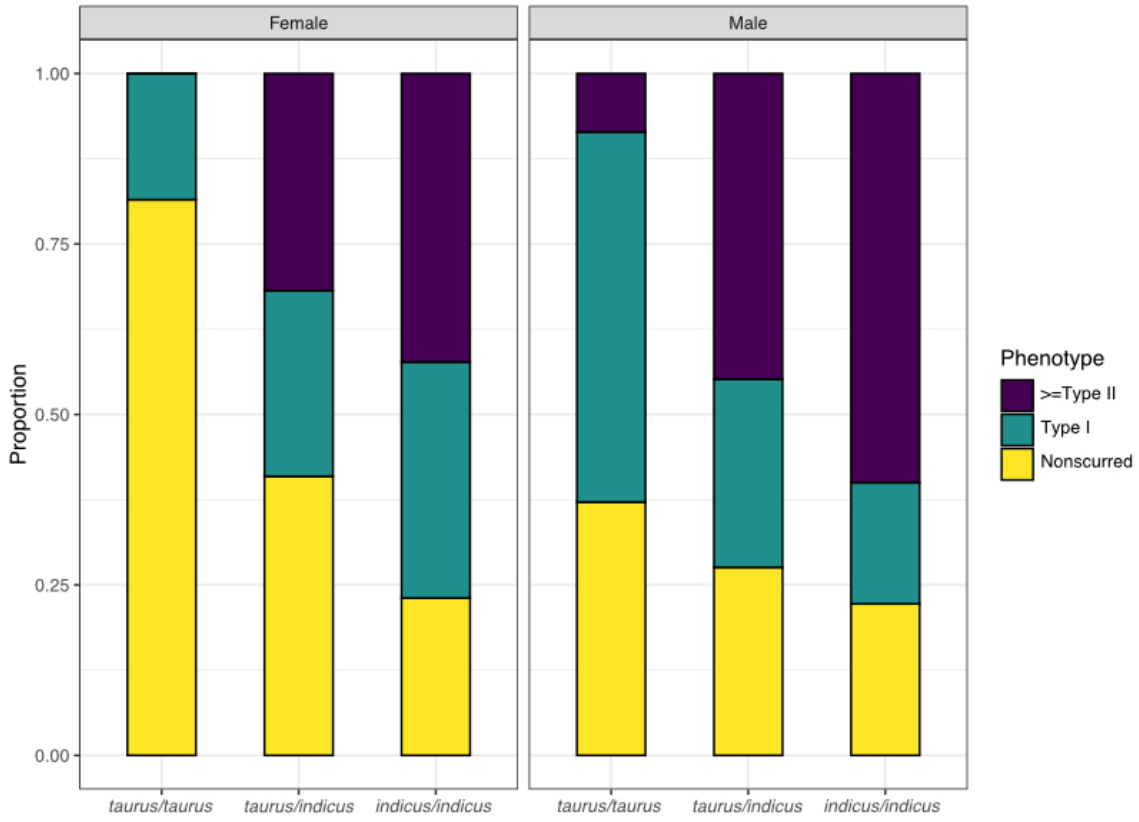

Figure 15 Phenotype distribution by subspecies-of-origin of genotypes in the *RXFP2* region. *taurus/taurus* = both haplotypes of *Bos taurus* origin; *taurus/indicus* = one haplotype of *Bos taurus* and one of *Bos indicus* origin; *indicus/indicus* = both haplotypes of *Bos indicus* origin. Only heterozygous polled cattle were included.

Identification of three Bos indicus insertions/deletions at RXFP2

To pinpoint potential causal mutations, we manually searched through sequence data for the *RXFP2* region of the 11 founders of McGregor population. Centered on the gene body of *RXFP2*, we designed primers for 10 putative insertions/deletions (InDels) that were different in at least one *Bos taurus* or *Bos indicus* founder. A PCR assay was carried out for a subset of families from the McGregor and Angleton populations, and we were able to locate 3 non-coding InDels that were in perfect association with breed-of-origin among the 7 families from McGregor we tested (family 70-77), with one at the 5' end of *RXFP2*, one at 3' end of *RXFP2* and an intronic InDel inside the gene body of *RXFP2* (Table 11). This suggests that all mutations (including SNPs and other types of mutations) between the 5' InDel and 3' InDel of *RXFP2* likely show subspecies differences in terms of the frequency of these mutations. In other words, the region of *RXFP2* was likely under different selection pressure in *Bos taurus* and *Bos indicus* cattle. In Angus, the selection pressure applied to the elimination of horned cattle may have extended to all types of headgear and changed the allele frequency in the region of *RXFP2*.

Table 11 Genomic position of three subspecies-specific InDels around *RXFP2*

Tag	Genomic position (ARS-UCD1.2)	Type
3D1	BTA12:29,200,690-29,200,880	Deletion in *Bos indicus* genome
I1	BTA12:29,268,148-29,268,280	Deletion in *Bos indicus* genome
5U1	BTA12:29,404,536-29,404,639	Deletion in *Bos indicus* genome

Predicting the effects of subspecies-specific InDels on genomic re-arrangements

As our understanding of the regulation of genomic features improves, we now know that non-coding structural variations (SVs) usually interrupt chromosome folding and thus affect the activation or repression of gene expression locally (Beagan and Phillips-Cremins, 2020; Szabo et al., 2020). Capture of the three-dimensional genome can provide such information, but it is also a relatively expensive technique and requires enormous computational resources. Therefore, to examine the genomic re-arrangement differences between *Bos taurus taurus* and *Bos taurus indicus* at *RXFP2*, we predicted the genomic folding structure for the *RXFP2* region. We found that although both subspecies are similar at this region, the local interaction points were predicted to be different, especially around the *RXFP2* gene body (Figure 16). This result is consistent with our findings of subspecies-specific InDels as InDels usually have profound effects on chromosome folding. This means such differences could enable the activation or inactivation of the expression of *RXFP2*, or different co-expression networks of the genes locally, due to the formation of different topologically associating domains (TADs).

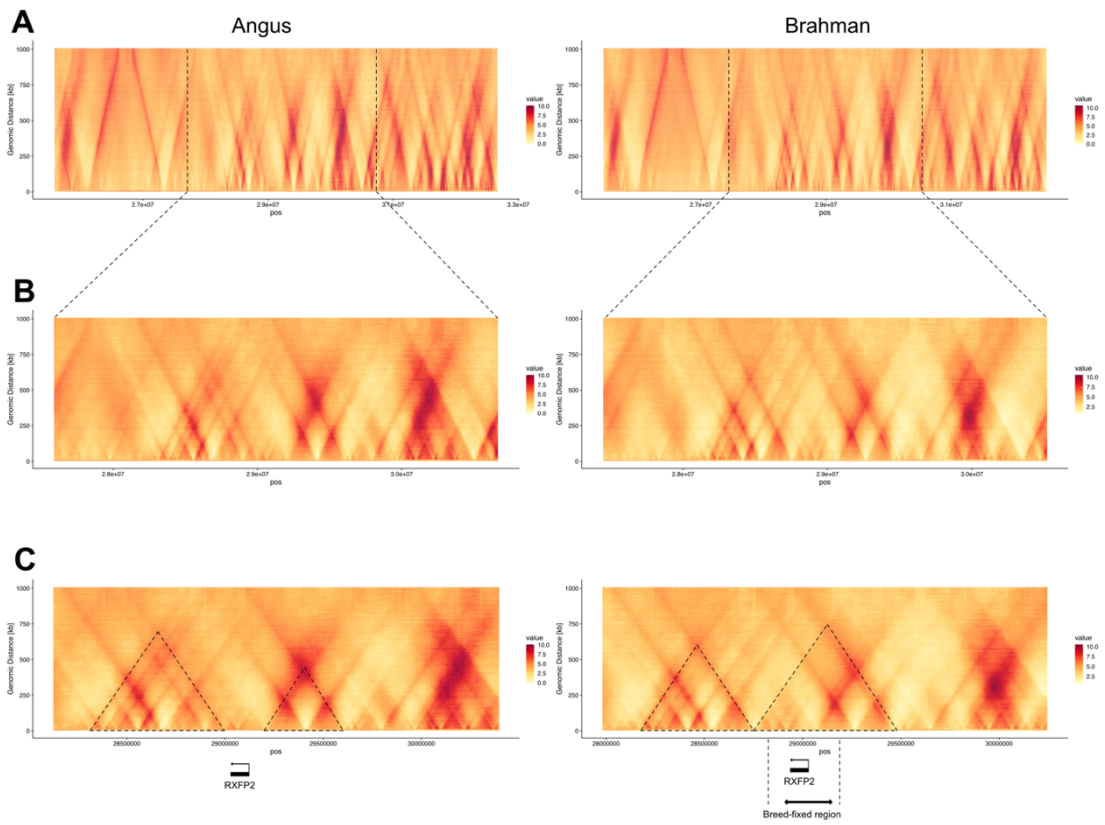

Figure 16 Heatmap of predicted genomic re-arrangements based on DNA sequences of *Bos taurus* and *Bos indicus*. Left panel is based on comparison to Angus and right panel is based on Brahman. (A) Genomic re-arrangements predicted for a 10 Mb window centered on RXFP2. (B) Zoomed-in prediction of genomic re-arrangements centered on RXFP2. (C) Annotated prediction of genomic re-arrangements for this region. Potential contact points were colored, and dotted line represents predicted Topologically associating Domains (TADs).

CHAPTER V

TRANSCRIPTIONAL CELL ATLAS FOR HEADGEAR IN CATTLE

Introduction

Both scurs and horns differentiate from neural crest cells in the same area of the frontal skull (Dove, 1935). In addition to having the same origin, the development of large scurs is very similar histologically to the development of horns. Although scurs usually develop later (Nasoori, 2020), previous histological studies have confirmed that the tissues at the site of horn buds of scurred and horned cattle undergo a similar process (Wiener et al., 2015). Briefly, at an early developmental stage, horned cattle were found to have glandular differentiation, aggregation of vacuolated keratinocytes in the epidermis, formation of nerve bundles and an absence of hair follicles. Similar characteristics can be found in scurred cattle at a much later stage, and as we described in Chapter II, different types of scurs develop features that belong to true horns. Polled cattle do not show signs of development of these features and no histological differences were found between skin from the site of horn buds and frontal skin.

Because of the resemblance between different types of scurs and true horns, the genetic connection between scurs and horns was the rationale for this final study. In fact, the relationship between scurs and horns has previously been discussed (Johnston et al., 2013; Pan et al., 2018; Randhawa et al., 2019) for other ruminants such as sheep that also develop scurs that do not fuse with the frontal skull (Johnston et al., 2013; Wang et al., 2019). Although sheep seem to maintain the development of their headgear with one

locus, *RXFP2* (Allais-Bonnet et al., 2013; Johnston et al., 2013; Wang et al., 2014; Pan et al., 2018), the same gene we identified in Chapter IV, the genetic mechanisms behind the morphology in sheep suggests the scurs and horns of Bovidae share the same pathway. Therefore, it becomes our natural next step to study the genetic relationship between scurs and horns in cattle.

Transcriptional profiles can be used as a direct tool to study the genetic relationship between scurs and horns. Previous studies about scurs and horns in cattle were limited to histology, real-time PCR, and genome-wide association studies (Capitan et al., 2011; Wiedemar et al., 2014; Tetens et al., 2015; Wiener et al., 2015; Gehrke et al., 2020). A lack of whole-genome transcriptional profiling for scurs and horns limits our understanding of the similarities and differences between the development of horns and scurs. Although traditional bulk whole-genome RNA-seq can provide a glimpse into the genome-wide dynamic expression network, challenges such as not being able to distinguish noise from untargeted cells make it less usable to study developmental biological problems like the growth of headgear, whose origin of differentiation is not clear.

Therefore, to study the genome-wide expression network of developing scurs and horns, while also being able to tackle the challenge that the development origin of headgear is not clear, we used spatial transcriptomics (ST), a newly developed technique that enables the investigation of whole-genome transcription while also retaining tissue context (Rao et al., 2021). Spatial transcriptomics can provide expression profiles at a near-single-cell resolution, combined with histology of the same region.

The objective of this chapter is to study the cellular expression network of developing scurs and horns.

Methods

Animal selection

All animal protocols used in this study were approved by the Texas A&M University Agriculture Animal Care and Use Committee.

For this chapter, cattle (~4 mo old) from Cycle 5 of the McGregor Genomics population were used. Before taking biopsies, we designed a PCR assay flanking the Celtic polled mutation (Wiedemar et al., 2014) to directly genotype the polled locus. We genotyped DNA extracted from 85 calves and 12 candidates for biopsy collection were selected based on the results from the PCR assay and written records of their horn status.

Sample collection

The status of horns for calves from the list of candidates was visually checked upon arrival at the McGregor Research Station in McGregor, TX. Photographs were taken of each calf showing the horn bud region of the skull. Biopsies from both sides of the head of these calves were collected using a tube dehorner.

After biopsies were taken, the tissue blocks were immediately transferred to a petri dish with pre-cooled PBS (Invitrogen, CA, USA). Tissue blocks were washed with cold PBS 3 times before they were cut into 2 pieces using a clean, cold razor blade. One of the 2 pieces was cleaned using paper towel to remove extra liquid on the surface and immediately put into liquid nitrogen wrapped in aluminum foil. The other piece was

also cleaned with paper towel to remove extra liquid on the surface and was then carefully sliced to prepare a thinner tissue block showing the vertical surface of the horn tissue. The sliced block was then transferred to a mold containing O.C.T. (optimal cutting temperature) solution (Fisher Scientific, USA) and placed on dry ice. After the mold was fully frozen, it was then wrapped with aluminum foil and transferred to liquid nitrogen immediately. Tissue blocks were then transferred back to the Kleberg Center and stored at -80°C.

Preparing tissue blocks for spatial transcriptomics

The 10X Genomics (Santa Monica, CA, USA) Visium Spatial Gene Expression platform (Rao et al., 2020) was chosen for this study. Briefly, tissue blocks were sliced into sections at a 10 μM thickness on a Leica CM1950 cryostat. Sections were then fixed on the Tissue Optimization Slide immediately inside the cold chamber of the cryostat following the tissue fixation protocol provided by 10X Genomics. A total of 7 sections were fixed on the Tissue Optimization Slide along with a blank spot as a negative control. The Tissue Optimization Slide was transferred on dry ice to the Texas A&M Institute for Genome Sciences and Society (TIGSS) Genomics Core, who performed the optimization procedure. The TIGSS Genomics Core tested a series of parameters such as the optimal permeabilization time and cDNA synthesis conditions following the standard optimization protocol provided by 10X Genomics. For this study, hematoxylin and eosin (H&E) staining was chosen to investigate tissue structure.

Once optimal conditions were determined, an expression slide with 4 section spots was prepared using the same tissue fixation procedure and submitted to TIGSS for

H&E staining, sequencing library construction and sequencing. The 4 section spots were assigned to horn buds collected from one polled steer (582J), one scurred steer (717J) with type II scurs, one scurred heifer (586J) with type I scurs and a horned steer (676J). Procedures for preparing the expression slide were the same as for the Tissue Optimization Slide except that there was no negative control spot and only 4 spots were on the expression slide. All procedures were done under a cold environment to ensure the integrity of RNA.

Library construction and sequencing

This procedure was performed by TIGSS. Briefly, permeabilized RNA molecules were captured by pre-fixed oligo probes on the slide. Each probe has a unique identifier sequence followed by an adaptor for cDNA synthesis. Captured RNA molecules were later supplied to cDNA synthesis under the optimal parameters obtained through the Tissue Optimization stage. Once the cDNA synthesis was completed, they were sequenced on an Illumina NextSeq using a 100 bp paired-end strategy.

Data analysis

Raw sequences were firstly grouped by lane, and demultiplexed using Space Ranger (v1.3.0), a custom software suite developed by 10X Genomics, to generate ready-to-use sequencing data. This step utilized the native Illumina *mkfastq* function and is customized for the 10X Genomics Visium Gene Expression platform.

A custom reference index for read alignment and gene expression quantification was then built from the ARS-UCD1.2 build of the cattle reference genome. A FASTA file and gene annotation file (GTF) of the ARS-UCD1.2 assembly were downloaded

from Ensembl. The GTF file was filtered using pre-built function *mkgtf* from Space Ranger to filter out unwanted features. For this study, retained features include protein coding genes, long noncoding RNAs, immune-related genes (Ensembl-coded "TR_" genes), small nucleolar RNAs and small nuclear RNAs. The filtered GTF file was then supplied to the pre-built function *mkref* to make a custom reference genome index for downstream read alignment and quantification of gene expression.

Space Ranger in combination with Loupe (10X Genomics) has a group of functions to map reads to the reference genome and automatically parse the histology images and align them with sequencing results. To achieve the best accuracy of alignment between sequencing data and histology staining, histology images were manually aligned using Loupe (10X Genomics). Image alignment parameters were then supplied to Space Ranger by specifying manual alignment flag "-*alignment*" so this step did not utilize the built-in automatic alignment algorithm, which may cause mismatches regarding the spatial information.

Mapped reads were automatically processed to produce a set of files containing information including gene expression quantification for each probe spot. Visualization of results was primarily done by Loupe. By default, Loupe automatically calculated spot clustering based on their expression profiles. Uniform Manifold Approximation and Projection (UMAP) for data dimension reduction was chosen to present the clustering results (Kobak and Berens, 2019; Linderman et al., 2019). It also provides functions such as total unique molecule identifier (UMI) count, total expressed gene count and

individual gene expression count for each spot. A custom R script was also used to generate visualizations for results.

Investigation of gene expression of regions of interest

To validate the previous GWAS results and focus on the genomic regions that are strongly associated with the development of horns and scurs, genes within the Celtic polled region as well as the genes uncovered in the previous chapter were manually investigated.

Comparisons between polled, scurred, and horned sections were carried out by combining the expression of genes within the region of the polled locus and *RXFP2* with genomic re-arrangements identified in the previous chapter. The correlation between predicted TADs and the expression of genes inside can thus be investigated and serve as a cross-validation of the predictions.

Results

Histology staining of polled, scurred and horned tissues

A total of 6 tissues samples collected from the regions where horns develop from two polled calves, two scurred calves and two horned calves, a female and male for each category, were submitted to Texas A&M Histology Lab for H&E staining.

For the polled calves, it is obvious that the samples we took are no different than a typical skin biopsy. We observed a significant number of hair follicles in the dermal layer while most of the connective tissue consisted of collagen. We did not observe the presence of nerve bundles (Figures 17).

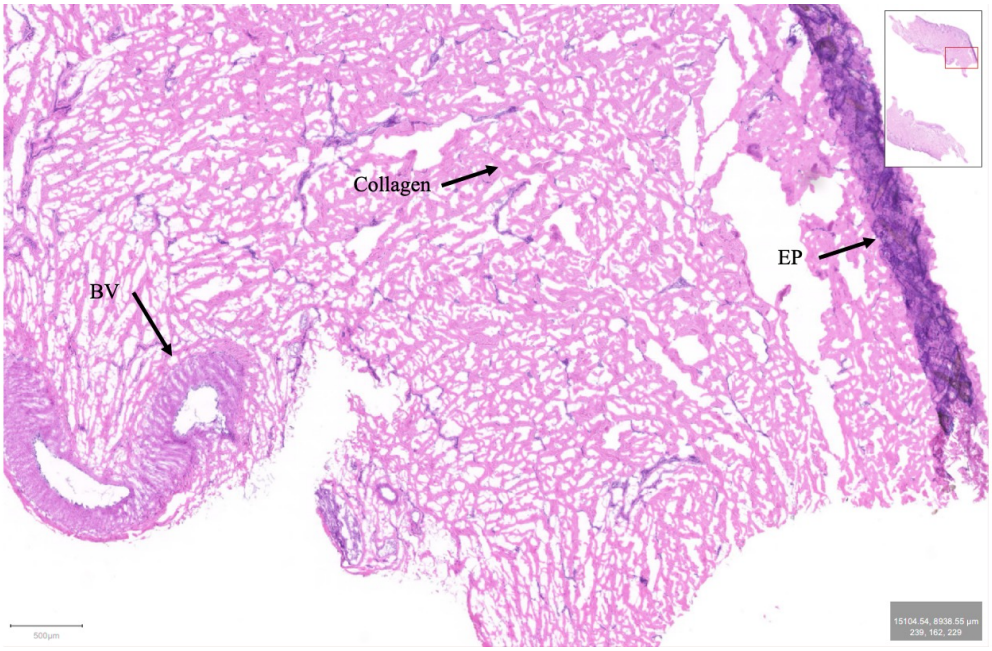

Figure 17 Frozen H&E section of the horn bud region of 582J (polled male). BV – blood vessel; EP – epidermis.

For the scurred calves, we found substantial differences between the male type II scurs (Figure 18) and female type I scurs (Figure 19). The biggest difference was the thickness of the epidermis. The epidermis of the scurred female was thicker than the scurred male, but both samples had a thicker epidermis than the polled samples. Although we also observed many vacuolated keratinocytes in both individuals, the male sample had an aggregation of a thick layer of keratin, whereas the female sample did not. In contrast to the polled sample, hair follicles were completely absent and nerve bundles were present in both individuals. One of the unique findings was that both male and female scurred calves showed an abnormal aggregation of inflammatory cells in the dermal tissue close to the epidermis.

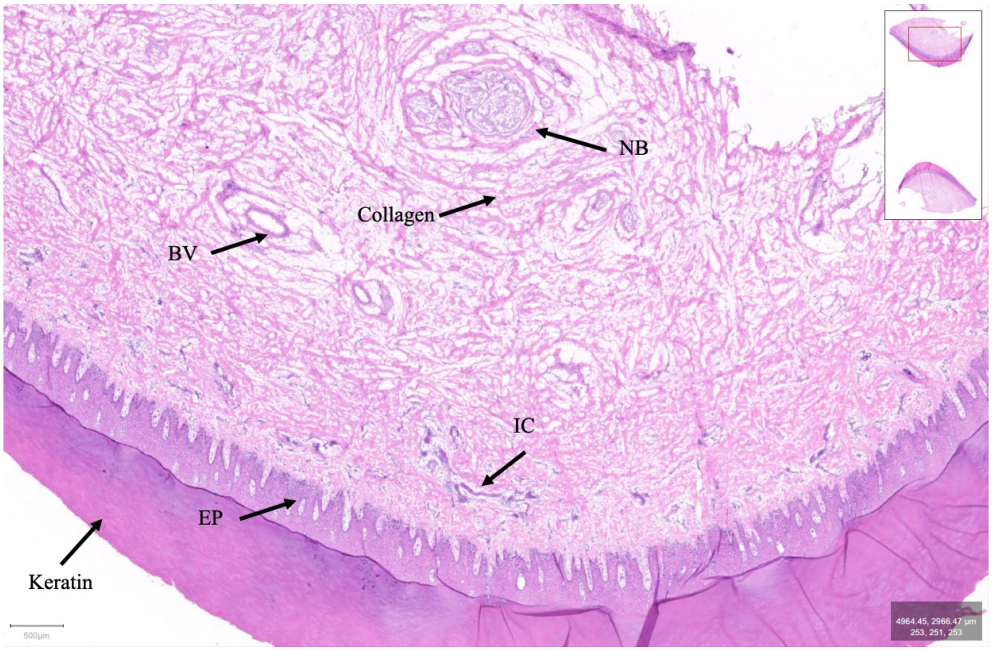

Figure 18 Frozen H&E section of the horn bud region of 717J (type II scurred male). EP – epidermis; IC – inflammatory cells; BV – blood vessel; NB – nerve bundle.

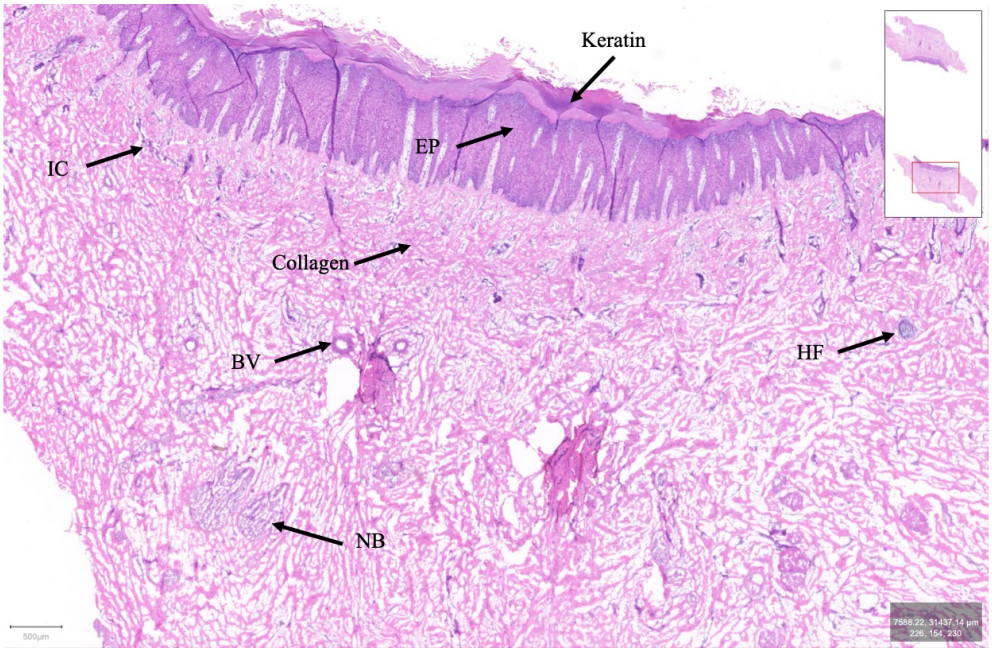

Figure 19 Frozen H&E section of the horn bud region of 586J (type I scurred female). EP – epidermis; IC – inflammatory cells; BV – blood vessel; NB – nerve bundle; HF – hair follicle (immature).

One thing to note is that because the 2 female scurred calves we sampled developed scurs similar to type I scurs and the 2 male scurred calves developed scurs similar to type II scurs, it is uncertain whether the differences we saw in histology are reflecting sexual dimorphism or the differences between a type I scur and type II scur.

It is challenging to compare horned samples (Figure 20) to the other two phenotypes because as mentioned above, horns tend to develop a lot earlier than scurs, and by the time we sampled these cattle, shaped horns were already present, and ossification had started in both horned cattle. We were only able to sample the peripheral region of the base of the horn, which we believe will differentiate as part of the horns as horns outgrow.

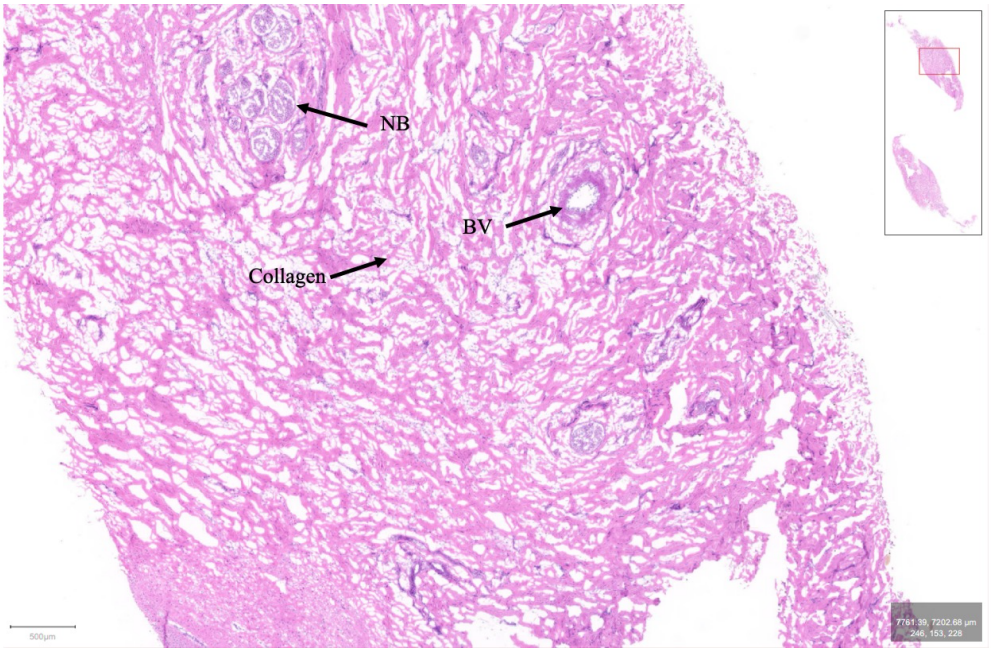

Figure 20 Frozen H&E section of the peripheral horn bud region of 676J (horned male). NB – nerve bundle; BV – blood vessel.

In this region, we observed a reduced number of hair follicles along with the formation of nerve bundles in connective tissue and aggregation of inflammatory cells. We were not able to obtain an intact layer of the epidermis, due to the fact the sample was peripheral to the base of the horn.

Spatial transcriptomics quality check

For tissue optimization, runtime and permeabilization were sequentially tested on every Tissue Optimization Slide section and the yield of cDNA was evaluated visually to determine the best conditions. Routine quality checks were followed and provided by TIGSS to ensure the yield of RNA of each tissue section was reasonable (Figure 21). After RNA molecules were captured and transcribed to cDNA using on-slide probes, fluorescence was used for RNA detection and each section was manually reviewed prior to sequencing.

Visualizations of total UMI and expressed genes per spot showed that low gene expression was detected for regions mainly consisting of collagen and regions close to the epidermis had the highest level of gene expression in terms of UMI and expressed gene count (Figures 22 and 23). This is consistent with histological observations where most of these regions consisted of collagen cells for all the samples.

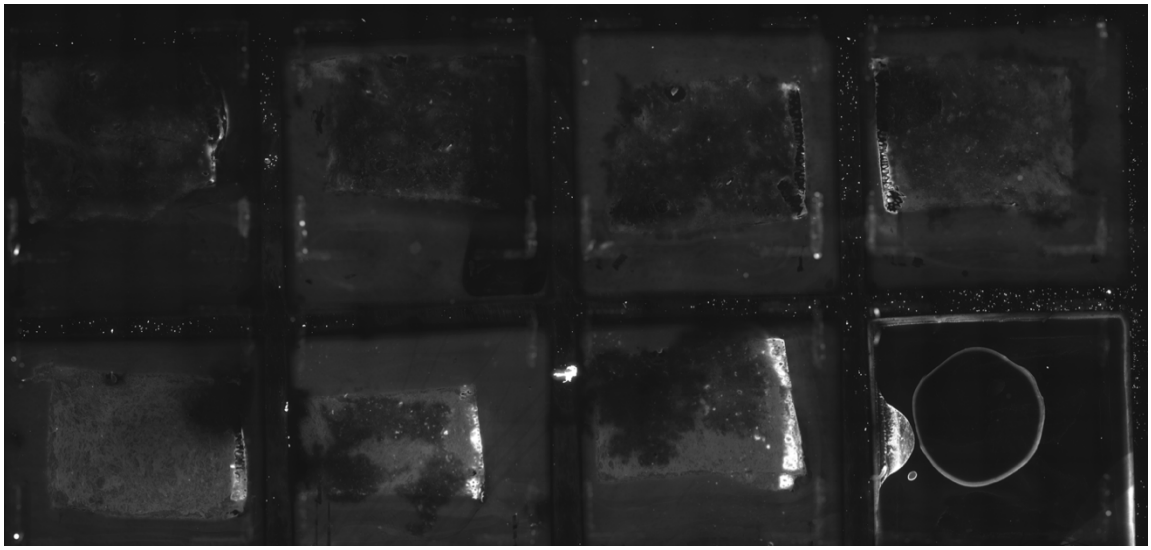

Figure 21 Fluorescence of cDNA synthesis under different settings using Tissue Optimization Slide. Each section represents a tissue section, and the lower-right corner section is for control purposes (no tissue section placed). The brightness represents the total cDNA yield for the region it represents. Each section was set under a series of different running time (provided by 10X Genomics) to determine the best parameters for horn bud tissues.

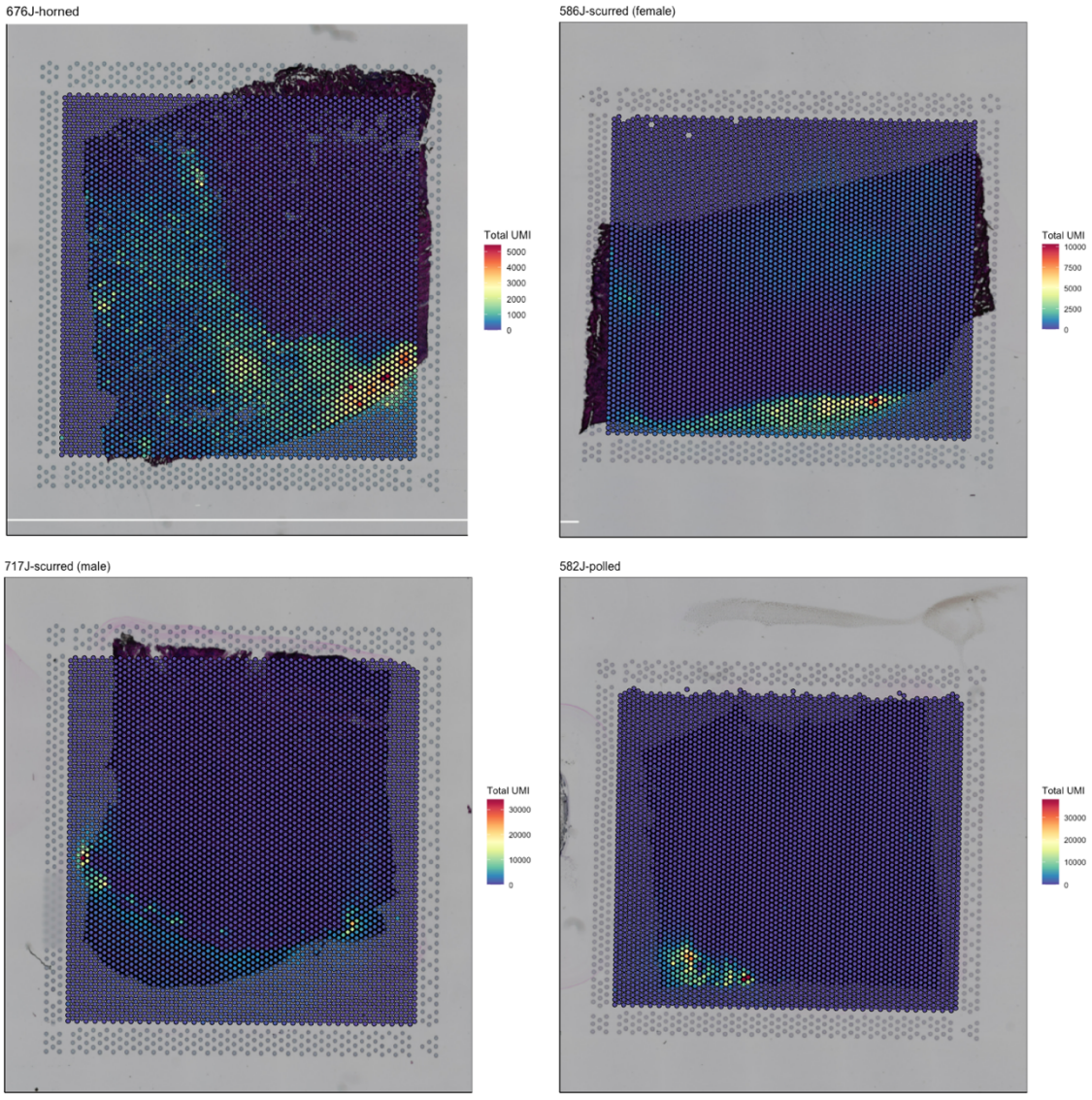

Figure 22 Total unique molecular identifier (UMI) detected for each spot for spatial transcriptomics

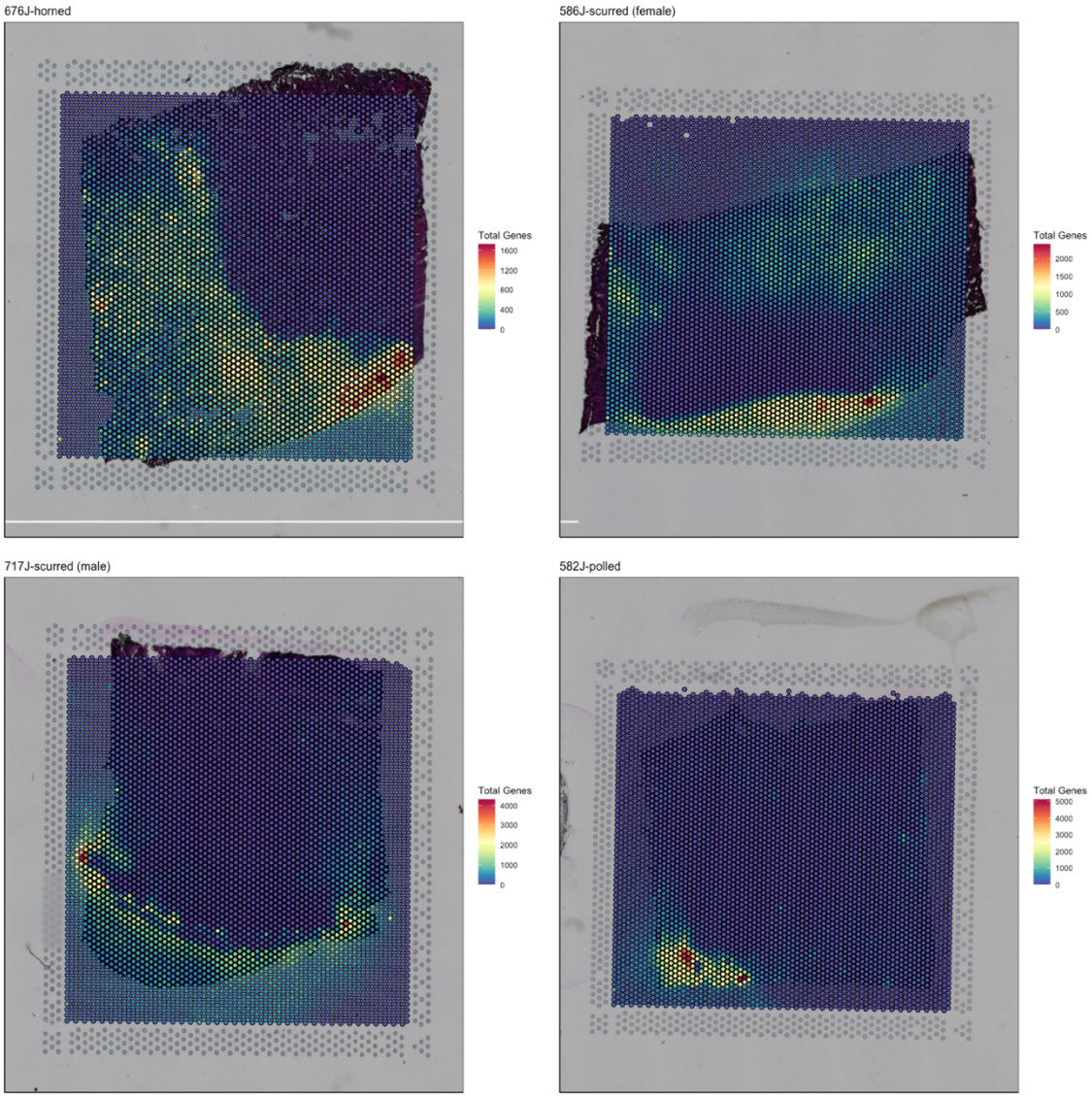

Figure 23 Total expressed genes detected per spot for spatial transcriptomics

Cell clustering based on Spatial Transcriptomics

Due to the nature of the Spatial Transcriptomics technique, clustering of expression profiles was done through the expression profiles of each capture spot, which covers on average of 4-5 cells per spot. Though performing profile clustering based on

capture spots may not generate the ideal resolution, it can still give us a picture of how different the cells are within polled, scurred and horned tissues.

As a result, we identified 4 clusters in the tissue from the polled calves. Different clusters of spots seem to be mixed in the polled calves, which is consistent with what we observed in the histology staining where no large structure was identified in the polled tissue (Figure 24).

We also identified 4 clusters for the male scurred tissue and 5 clusters for both horned and female scurred tissues. Although the number of clusters is similar among polled, scurred and horned tissue, clusters of spots in scurred and horned tissue seem to be more aggregated rather than spread across the entire tissue section. Structures like nerve bundle and epidermis in the scurred and horned tissues were correctly reflected by the expression profiles.

Our results also indicate that, regardless of whether the tissue was from a polled, scurred or horned calf, the region where a horn develops does not seem to contain a lot of different types of cells, and it can be inferred that the development of headgear in cattle at this stage is probably driven by only a few genes.

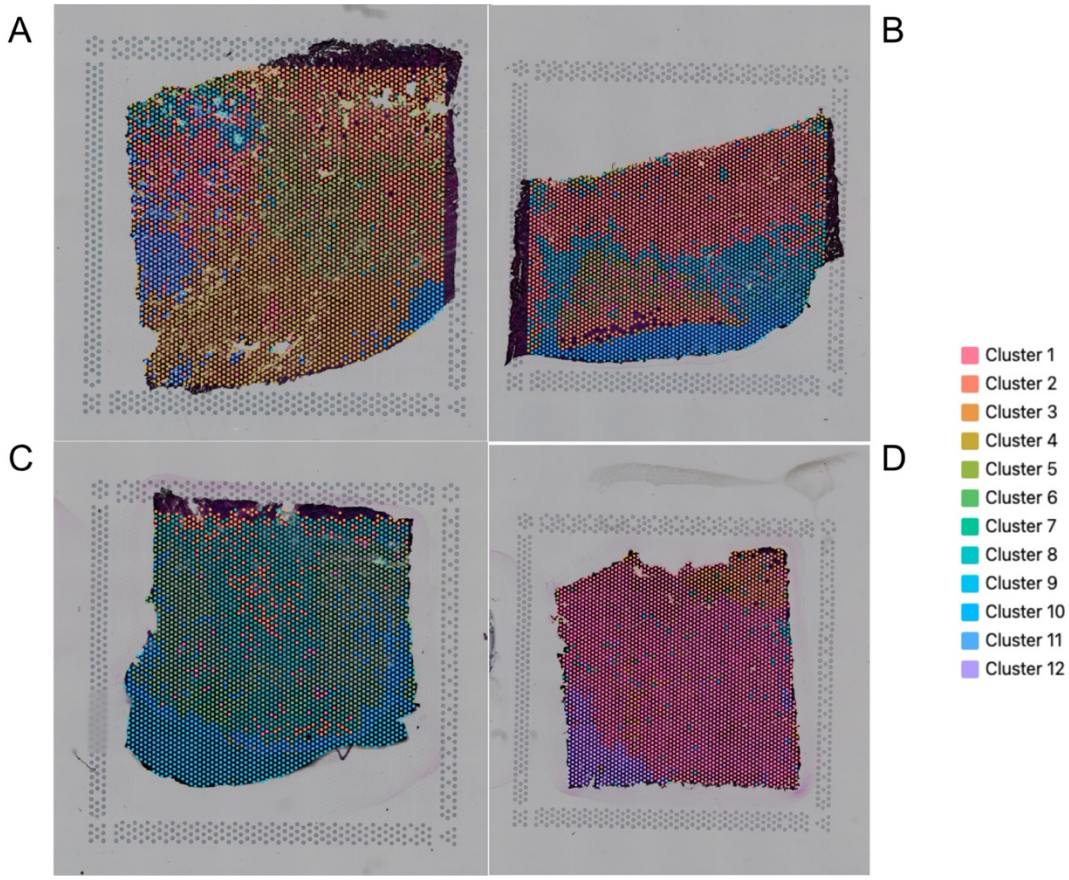

Figure 24 UMAP clustering of spatial transcriptomics. Each color represents a cluster based on expression profiles and colors are unified across all samples. (A) 676J (horned steer); (B) 586J (type I scurred heifer); (C) 717J (type II scurred steer); (D) 582J (heterozygous polled nonscurred steer)

Investigation of the regions of the Celtic polled locus and RXFP2 in spatial expression profiles

As we pointed out in Chapter IV, a major locus associated with the development of scurs in cattle is the well-studied headgear-related gene, *RXFP2*. We first directly compared the expression level of *RXFP2* between polled, scurred and horned tissue by visualizing its expression on spatial sections. We found that female scurred and peripheral horned tissue have significantly more *RXFP2*-expressed spots than the polled

tissue and male scurred tissue, as there were nearly no expressing spots at all (Figure 25). In contrast, we were not able to detect the expression of *RXFP1* in any of these sections.

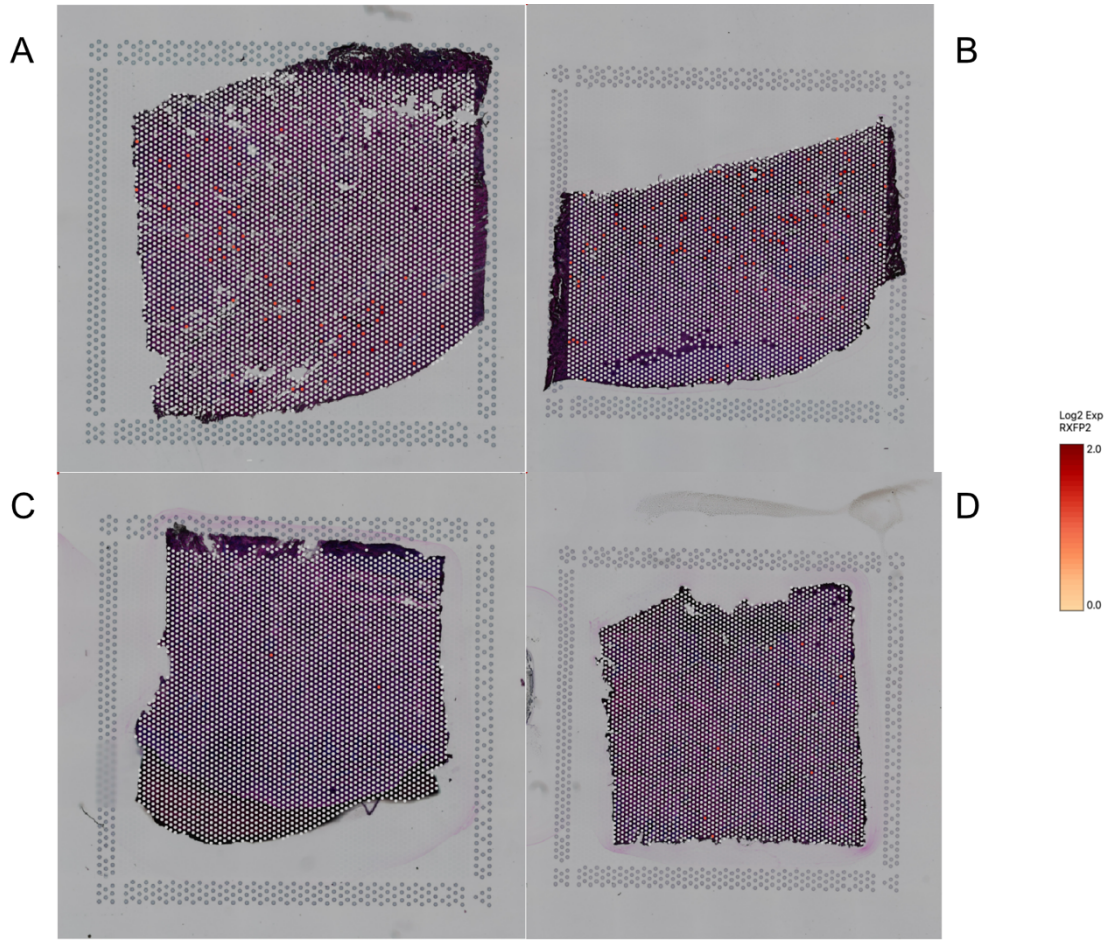

Figure 25 The expression pattern of *RXFP2* across sections. Each dot represents a capture area and color scheme represents the expression level of *RXFP2*. (A) 676J (horned steer); (B) 586J (type I scurred heifer); (C) 717J (type II scurred steer); (D) 582J (heterozygous polled nonscurred steer)

For *APPL1* which was mapped for males for the presence of sheath, we were not able to detect the expression of this gene in the nonscurred and type II scurred sections

(Appendix C), but expression was detected in the horned and type I scurred sections. This result suggests the expression of this gene shows similar pattern comparing to *RXFP2*.

The development of scurs in cattle cannot be discussed without considering the polled locus. Thus, we also investigated the expression of genes around the Celtic polled locus. We visualized the expression pattern of every gene within the polled interval and found a co-expression pattern of *IFNAR1*, *IFNAR2* and *IL10RB* in scurred and horned tissues, whereas other genes within this interval did not show a significant difference in expression from the polled tissue (Appendix C). To understand these results, we predicted the genomic re-arrangements of the polled interval and found that *IFNAR1* (BTA1:2,189,895-2,218,334), *IFNAR2* (BTA1:2,315,596-2,350,090) and *IL10RB* (BTA1:2,242,523-2,314,142) are predicted to be within the same topologically associating domain (Figure 26). This result suggests the expression of a key group of genes that play an important role in the development of horns was interrupted by the polled mutation, and for heterozygous polled cattle, the expression of this core was likely not affected.

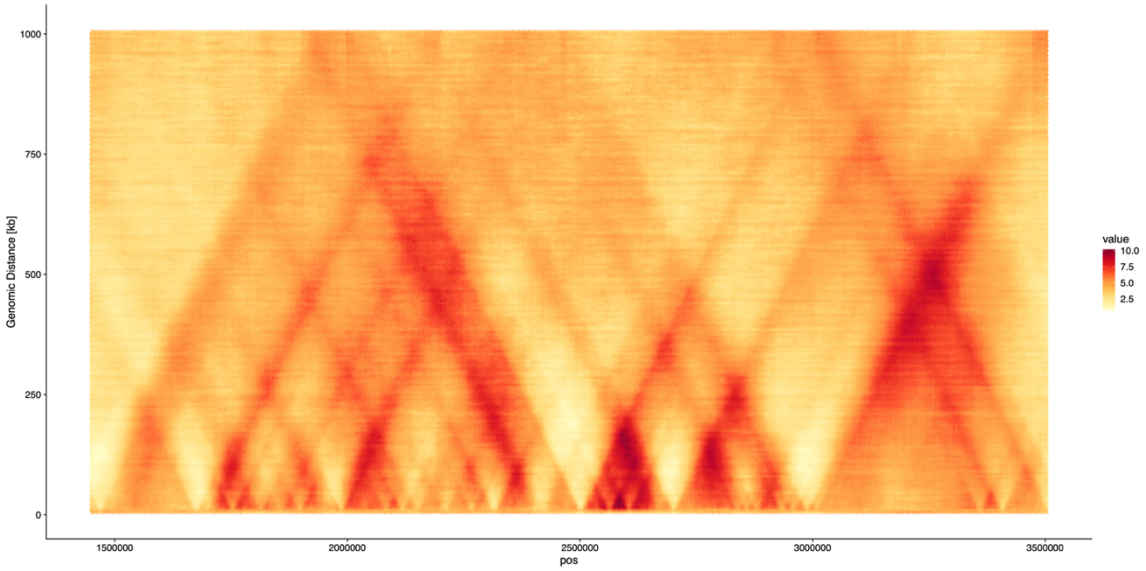

Figure 26 Predicted genomic rearrangements at the polled interval. The Celtic polled mutation is approximately mapped at BTA1:2480000-2500000.

CHAPTER VI

DISCUSSION AND CONCLUSION

Structural connection between scurs and horns

Previous studies that were designed to map the scurs locus ignored possible physiological connections between the scurs and horns phenotypes and treated all scurs as one category in the comparison (Asai et al., 2004; Capitan et al., 2009; Gehrke et al., 2020). Two justifications for this previous approach are 1) the morphology of scurs shows inconsistency in terms of appearance when compared to horns, and 2) genetic studies have established that the *scurs* locus must be an independent locus rather than a different allele at the *polled* locus (White and Ibsen, 1936; Long and Gregory, 1978; Capitan et al., 2009). These results suggested that the genetic basis for scurs is different from horns.

These two reasons can be challenged in their own way, based on what is already known about the development of horns in Bovidae. For cattle, development of horns starts at about day 70 of gestation (Wiener et al., 2015). Epidermis in the regions where horns grow becomes thicker and keratinocytes are observed (Li et al., 2018). It is agreed that there is no ossification (os cornu) of the horns and they remain as soft tissues before birth (Dove, 1935; Wiener et al., 2015). In some cases, the horn buds (initial protrusions) are identifiable upon birth as thicker, lightly keratinized skin due to the presence of keratinocytes (Dove, 1935). The horn buds then undergo a supposed epithelial-to-

mesenchymal transition where protrusions arise under the skin and form a pair of primitive cores beneath the skin (Allais-Bonnet et al., 2013).

From this point, it is thought that at least two parallel processes occur at the site of the horn buds (Dove, 1935). The first process starts with the skin. The epidermis keratinizes and becomes a hard integument and forms the tip and keratin sheath of the horns. Meanwhile, a pair of primitive cores within the dermis and connective tissue start to ossify and become the bony cores or horns. Bony cores fuse to the frontal skull through the frontal periosteum, and frontal sinus later invades into horns through the base of the horns.

Dove (1935) provided insights from a groundbreaking perspective about the formation of horns. Through dissection and tissue transplantation, he revealed the development process of bovine horns for the first time: 1) horns are dependent on the interaction between several tissue types including the epidermis, dermis, connective tissue, frontal periosteum and the frontal skull; 2) the origin of the os cornu for horns lies somewhere within the dermis and connective tissue as an independent process rather than an outgrowth of the frontal skull; 3) the formation of horns is a result of several parallel processes that do not induce or prohibit each other. These previous findings are important when we study scurs in cattle.

Based on our categorization, different types of scurs mimic several of the early stages of horns like Dove (1935) described. Type I scurs are similar to the beginning of horn formation in which soft protrusions thicken, and slightly keratinized skin is formed; type II scurs develop further, forming the keratin sheath; and, type III scurs have

developed all the structures seen in the previous two types of scurs while enabling the other parallel process where an os cornu inside starts to differentiate. These anatomic similarities show that scurs and horns are connected: scurs are early forms of horns except the fusion with the skull generally does not happen and pneumatization does not occur. The different types of scurs also suggest that there are genetic reasons why some scurs stop earlier in development than others.

Based on the results from H&E staining, the most noticeable results can be summarized as below:

1. There are no nerve bundles in the polled sample, which is consistent with a previous report (Wiener et al., 2015). The formation of nerve bundles suggests the horn region is more sensitive compared to the frontal skin of polled cattle.
2. Type I scurs differ from type II scurs in terms of the thickness of the epidermis as well as the accumulation of keratin above the epidermis. We speculate the formation of the shape of a type II scur is the result of a different keratin deposition ratio across the epidermis of this region.
3. We have observed an accumulation of inflammatory cells below the epidermis in both type I and type II scurs samples. Such unusual accumulation may suggest ongoing differentiation because stem cells employ immunoregulatory properties to maintain ontogenetic development and tissue homeostasis (Shi et al., 2015).

Inheritance of scurs

Early inheritance models for the presence or absence of scurs, such as the Long and Gregory (1978) model, were proposed based on phenotypic records. Long and Gregory (1978) proposed their model before the polled mutation was identified, thus genotypes at the polled and scurs loci could only be inferred from family pedigree and phenotypic records. Such inference of genotypes has a fundamental challenge: for scurs that have developed sufficiently, misclassification of a scur as a horn could occur and it could affect the inference of genotypes entirely. In fact, even Long and Gregory (1978) mentioned such a challenge. They have reported an exception to their proposed model where a putative *PpScsc* bull who produced scurred female progeny, was polled. Long and Gregory (1978) claimed that this was probably due to a misclassification and therefore dropped the case from their study.

Aside from the difficulties of precisely identifying scurs, the epistatic effects of the polled locus over the scurs locus hinder the information needed to determine the mode of inheritance. There are a few reasons why this matters:

1. Prior to the identification of polled mutations, it was difficult to study the genetic mechanisms of scurs using a random population, as a random population may not have enough segregation at the polled and scurs loci. Such a challenge can be seen in our study as well: the Angleton and McGregor populations contain over 3,000 cattle combined, but only 468 cattle were included in our final study due to the absence of phenotypic records and the homozygous state at the polled locus.

2. In previous studies (White et al., 1936; Long and Gregory, 1978; Capitan et al., 2009), generally the genotype of only one of the parents could be inferred because one or both parents were purebred *Bos taurus* or *Bos indicus* and from breeds that do not segregate at the polled locus. For example, Long and Gregory (1978) studied scurs based on progeny produced from an Angus x Hereford cross, whereas Capitan et al. (2009) studied the inheritance of scurs by mating non-scurred heterozygous polled French Charolais bulls to horned Charolais dams. Epistasis of the polled locus over the scurs locus hindered determination of the genotype at the scurs locus.

In our study, the McGregor population provided a unique opportunity to address these challenges as it is a *Bos taurus*-*Bos indicus* F_2 cross population. Parents from this population were all heterozygous polled and scurred. Additionally, although the Angleton population is a *Bos taurus*-*Bos indicus* backcross population, the F_1 parents were scurred as well. Brenneman et al. (1996) hypothesized that the allele for the presence of scurs is fixed in *Bos indicus* and the allele for the absence of scurs is fixed in *Bos taurus* because all F_1 parents were scurred. Although Brenneman et al. (1996) concluded this was not true and the Angus parents of the Angleton population were not fixed for the sc allele, their analyses were done before the polled locus was identified and misclassification of large scurs as horns could have influenced their conclusion.

Thus, by addressing the previous challenges, we re-evaluated the mode of inheritance for the presence or absence of scurs in a more precise way. Although we cannot reject a dominant mode of inheritance for the scurs phenotype in *Bos taurus* x

Bos indicus crosses, this result does not address the observation that more scurred males were found than females in both populations.

Spatial expression atlas of cattle headgear

We investigated the cellular gene expression atlas of polled, scurred and horned cattle using spatial transcriptomics. Spatial transcriptomics can provide insights of the expression profile while not losing tissue context (Burgess, 2019). For our study, in addition to comparing expression profiles, the tissue context associated with expression profiles added details about the cellular changes in the regions where the horn bud develops.

One of the challenges associated with spatial transcriptomics is to determine the optimal conditions for tissue permeabilization and cDNA synthesis (Ståhl et al., 2016). We have observed regions on these sections with a very low yield of mRNA, which may have a biological basis or may be a technical artefact because some regions were not fully permeabilized. As the major cell type within these sections is collagen, a low permeabilization ratio for certain regions is expected. Because collagen cells are usually large with no fixed shape (Majzoub et al., 2001), the yield of mRNA molecules was not expected to cover every capture spot. For regions with low mRNA yield, we also observed high expression of certain genes such as COX6, which is a skeletal muscle-specific gene. This suggests that although the yield of mRNA is low, there are still RNA molecules captured by the oligo tails on the spatial transcriptomics expression slide, and the patterns of expression observed may reflect biological differences among the tissues.

The expression atlas suggested that the composition of cells in the horn bud region is limited only to a few types of cells in terms of expression profiles, which is consistent with our observations of anatomy. There were differences in expression of the target gene, *RXFP2*, between the horned, polled and scurred samples. We have found a lack of expression of *RXFP2* in the polled frontal skin and type II scurs whereas type I scurs and the horned tissue both showed accumulated expression of *RXFP2*. Given that we sampled a region peripheral to the ossified horn in which we expect differentiation was still occurring, this observation of *RXFP2* expression in type I scurs and horns aligns with our GWAS results that *RXFP2* is needed more in the initiation stage of headgear development than later. These results suggest *RXFP2* is the scurs locus.

Although *RXFP2* was also mapped in males for the presence of sheath, it is more complicated to infer whether *RXFP2* is also the sheath locus. For example, the development of type I scurs in males may be compensated by another locus (such as *RXFP1,* which we mapped) and therefore both loci could be scurs loci. Since we did not identify any significant associations in females for the presence of sheath, *RXFP2* likely contributed more to the formation of sheath in males than females.

We also investigated the expression of genes at the polled locus. The polled locus is likely a master regulator of the development of headgear in cattle, as no headgear can grow in homozygous polled cattle. In horned cattle, genes in the pathway for development of scurs are active, and our results suggest scurs and horns share the same pathway. Expression patterns for *IFNAR1*, *IFNAR2* and *IL10RB* were similar in the type 1 scur, type 2 scur, and the horned sample, whereas the polled section did not show the

expression of these genes. However, *OLIG1*, a previously identified pecoran-specific gene under positive selection (Wang et al., 2019), was not different at this developmental stage (4 mo of age). The shared expression of *IFNAR1*, *IFNAR2* and *IL10RB* between both types of scurs and horns provided insights into our hypothesis that scurs and horns are connected. It provides evidence from the gene expression level that scurs have the same developmental origin as horns and the different types of scurs reflect the different stages of horn development affected by the associated mutations.

Combining the new knowledge we have generated regarding the inheritance of scurs, genes associated with the formation of scurs, and spatial expression patterns, we propose a new model of the genetic basis for scurs (Figure 27). Briefly, the signal for the development of headgear can be transduced in heterozygous polled cattle, but is blocked in homozygous polled cattle. The transduction signal binds with *RXFP2* in this region for the continuation of differentiation, where male cattle may use extra *RXFP1* to compensate the loss of function of *RXFP2*. The expression pattern for *APPL1* is similar to *RXFP2*, and previous research demonstrated that *APPL1* mediates adiponectin signal transduction in RANKL-induced osteoclastogenesis (Tu et al., 2011), making APPL1 and good candidate gene for a role in the formation of headgear. With the additive relationship between the *Bos indicus*-influenced haplotypes and scur morphology, we hypothesize that the morphology of scurs is dependent on dosage effects from *RXFP2*. On the other hand, for homozygous horned cattle, the homozygous state of the polled locus serves as a master regulator that enables horn development regardless of levels of expression of other loci, including *RXFP2*.

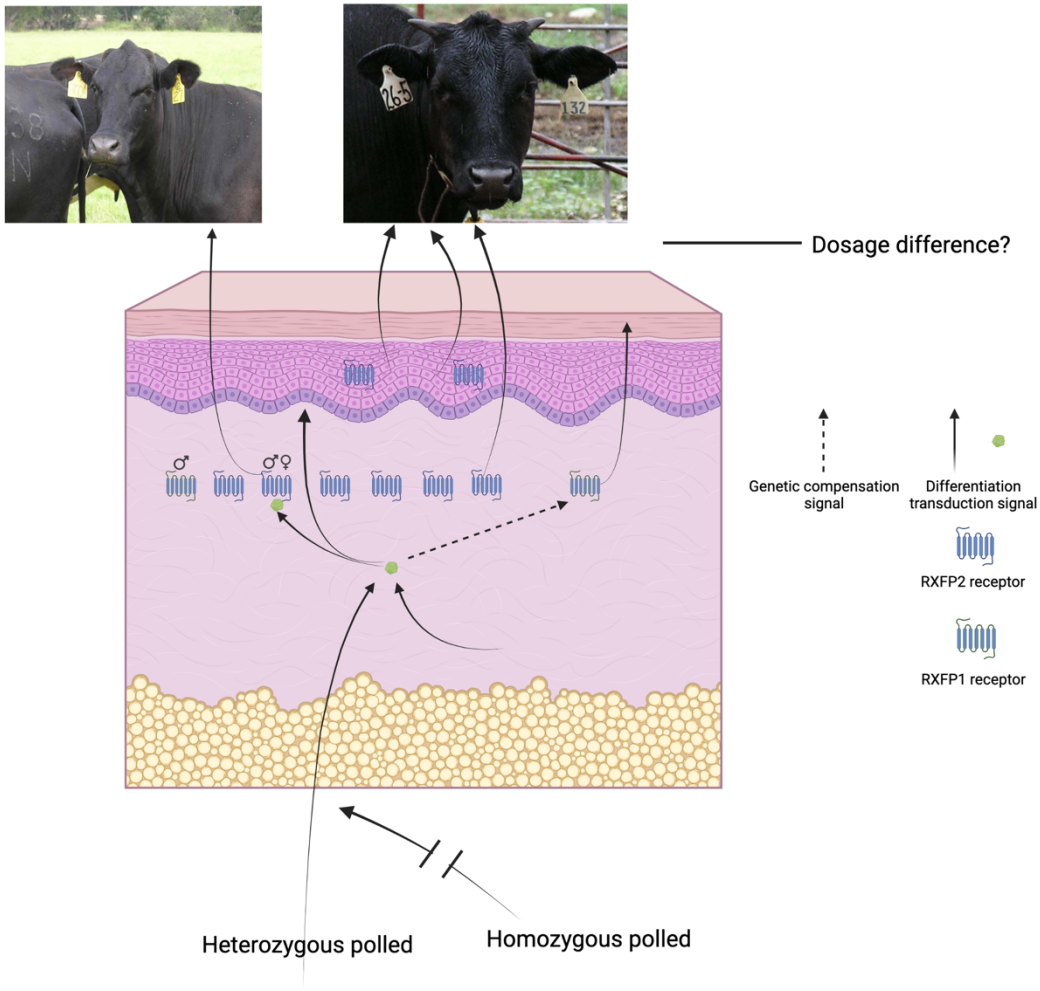

Figure 27 Genetic basis for cattle headgear. The polled locus is a master regulator and signal transduction in the dermis and epidermis is blocked in homozygous polled cattle. In heterozygotes, signal transduction is mediated by RXFP2 with additional compensation from RXFP1 in males.

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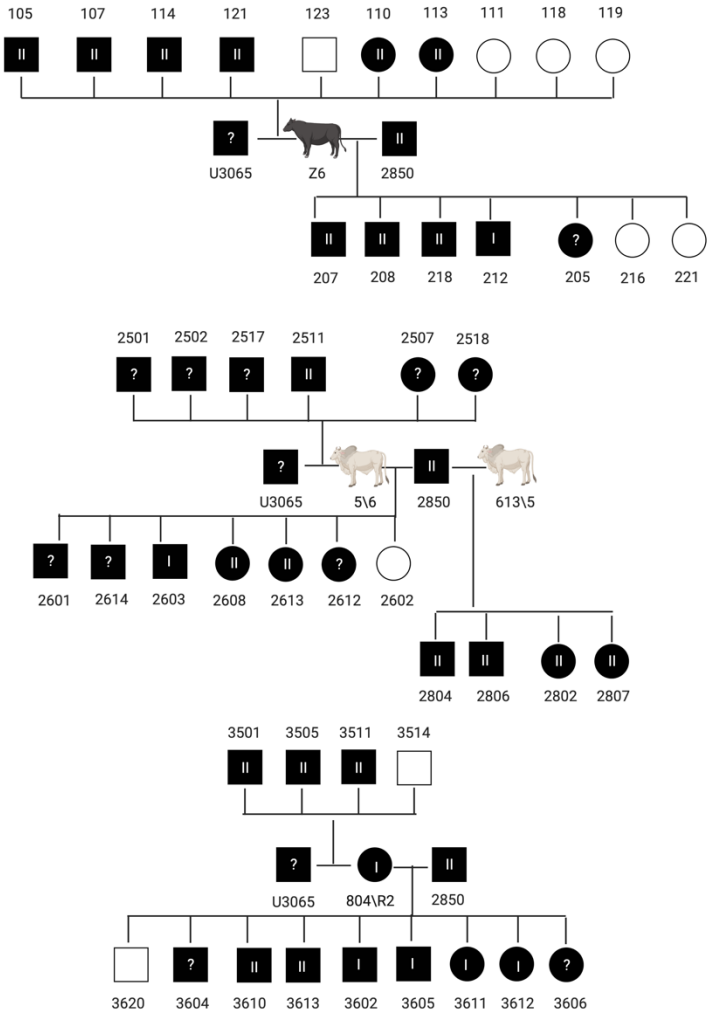
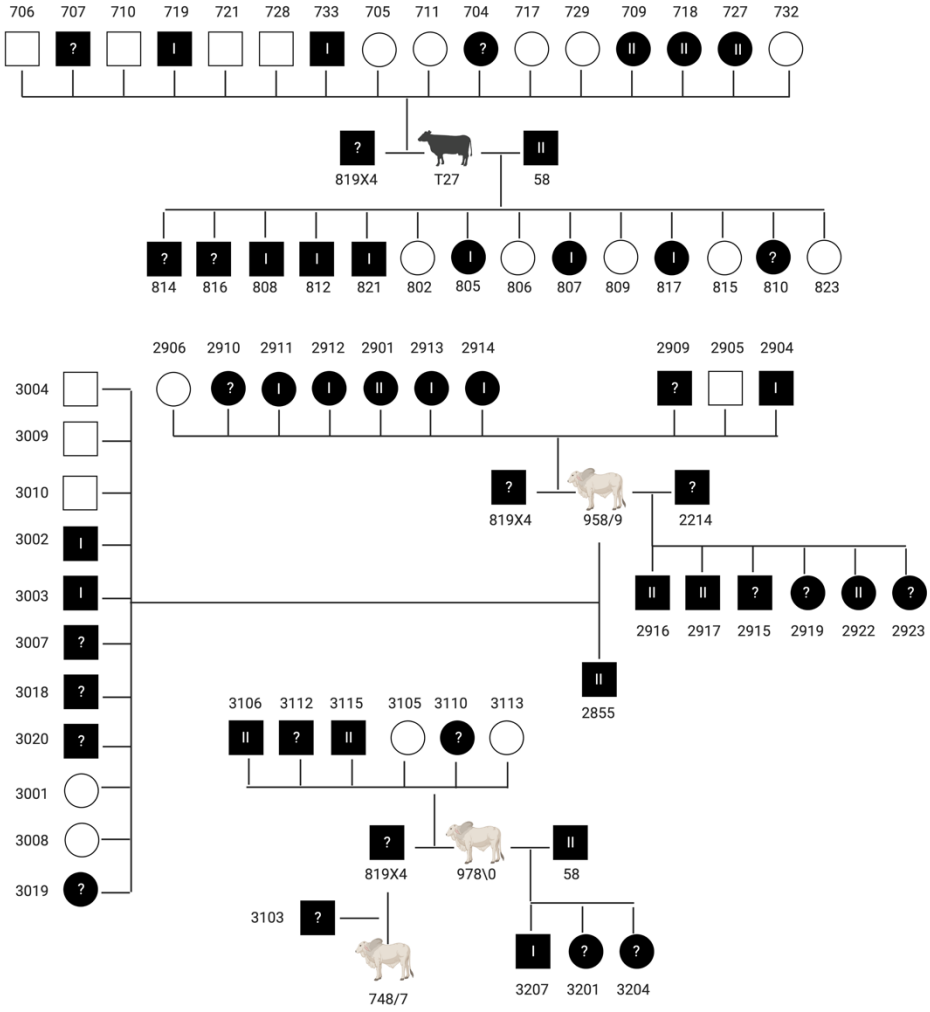
APPENDIX A

PEDIGREES OF THE ANGLETON AND MCGREGOR POPULATIONS

In each pedigree diagram only heterozygous polled progeny of the respective matings are shown. Squares are male, circles are female. White is nonscurred, black is scurred. I = type I scurs, II = type II scurs, III = type III scurs, ? = inconclusive.

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2906 2910 2911 2912 2901 2913 2914 2909 2905 2904
3004 3009 3010 3002 3003 3007 3018 3020 3001 3008 3019
819X4 958/9 2214
2916 2917 2915 2919 2922 2923
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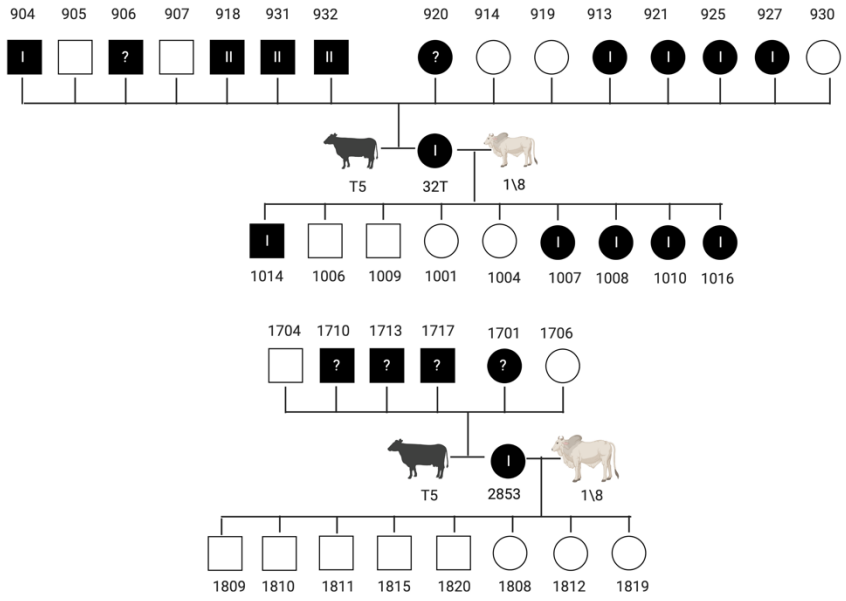
819X4 978\0 58
3103
748/7
3207 3201 3204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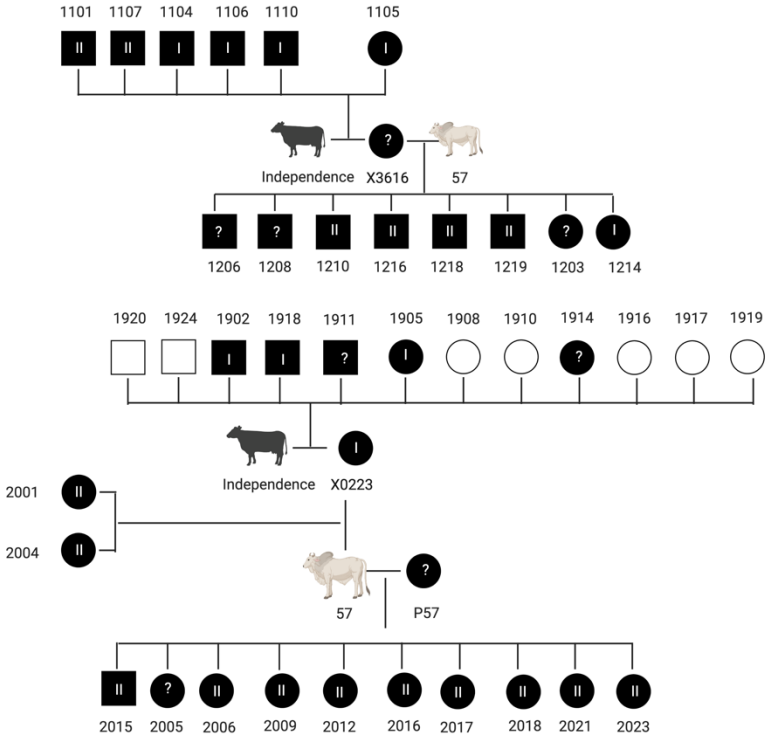
T5 32T 1\8
1014 1006 1009 1001 1004 1007 1008 1010 1016
1704 1710 1713 1717 1701 1706
T5 2853 1\8
1809 1810 1811 1815 1820 1808 1812 1819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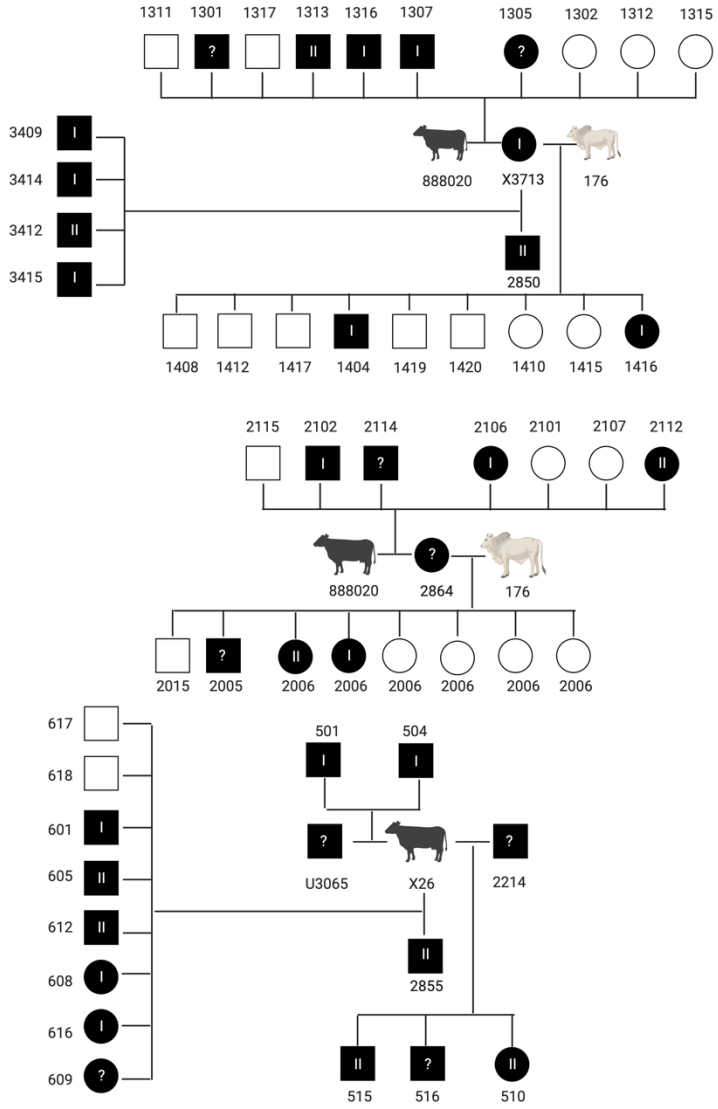
Independence X3616 57
1206 1208 1210 1216 1218 1219 1203 1214
1920 1924 1902 1918 1911 1905 1908 1910 1914 1916 1917 1919
Independence X0223
2001
2004
57 P57
2015 2005 2006 2009 2012 2016 2017 2018 2021 2023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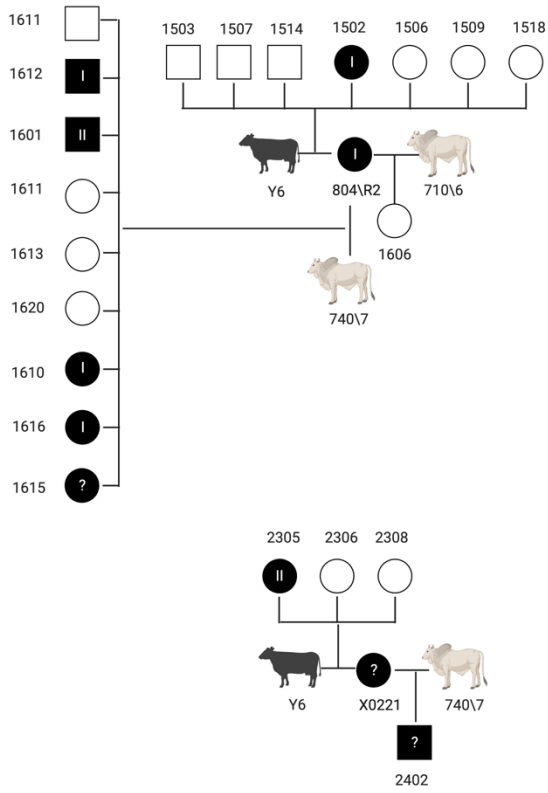
2855
515 516 510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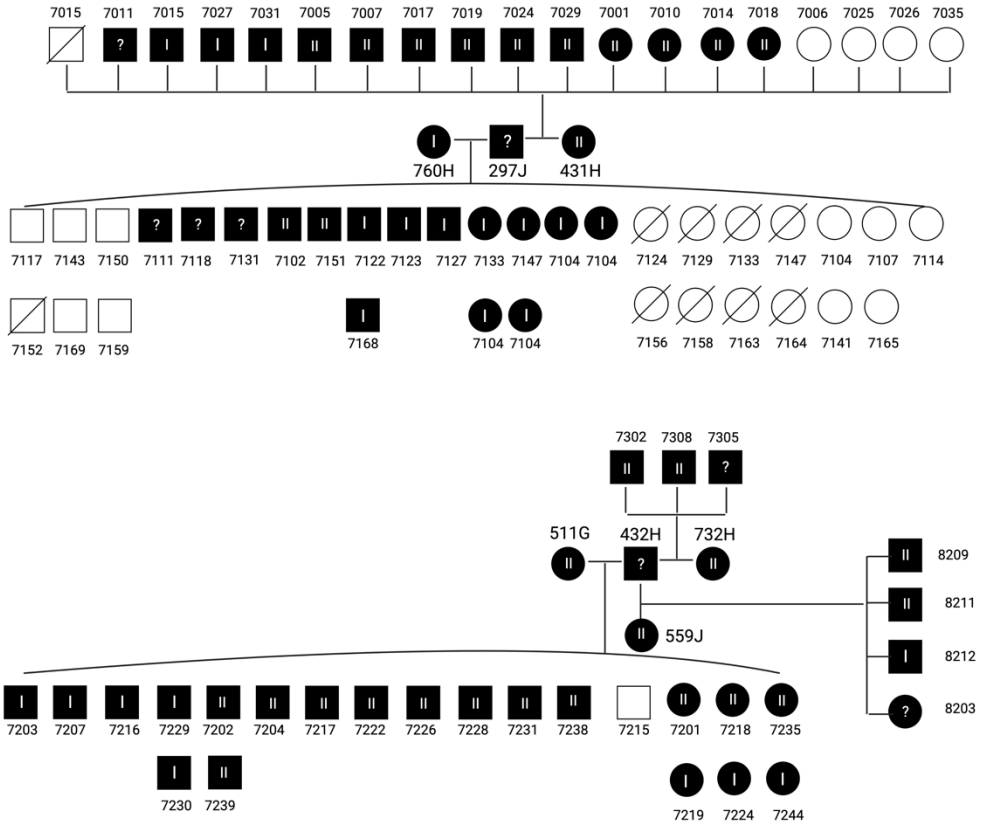
1601
1611
1613
1620
1610
1616
1615
I
II
I
I
?
1503
1507
1514
1502
1506
1509
1518
I
Y6
804\R2
710\6
1606
740\7
2305
2306
2308
II
Y6
?
X0221
740\7
?
2402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7152 7169 7159
7168
7104 7104
7156 7158 7163 7164 7141 7165
7302 7308 7305
511G 432H 732H
559J
8209
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8212
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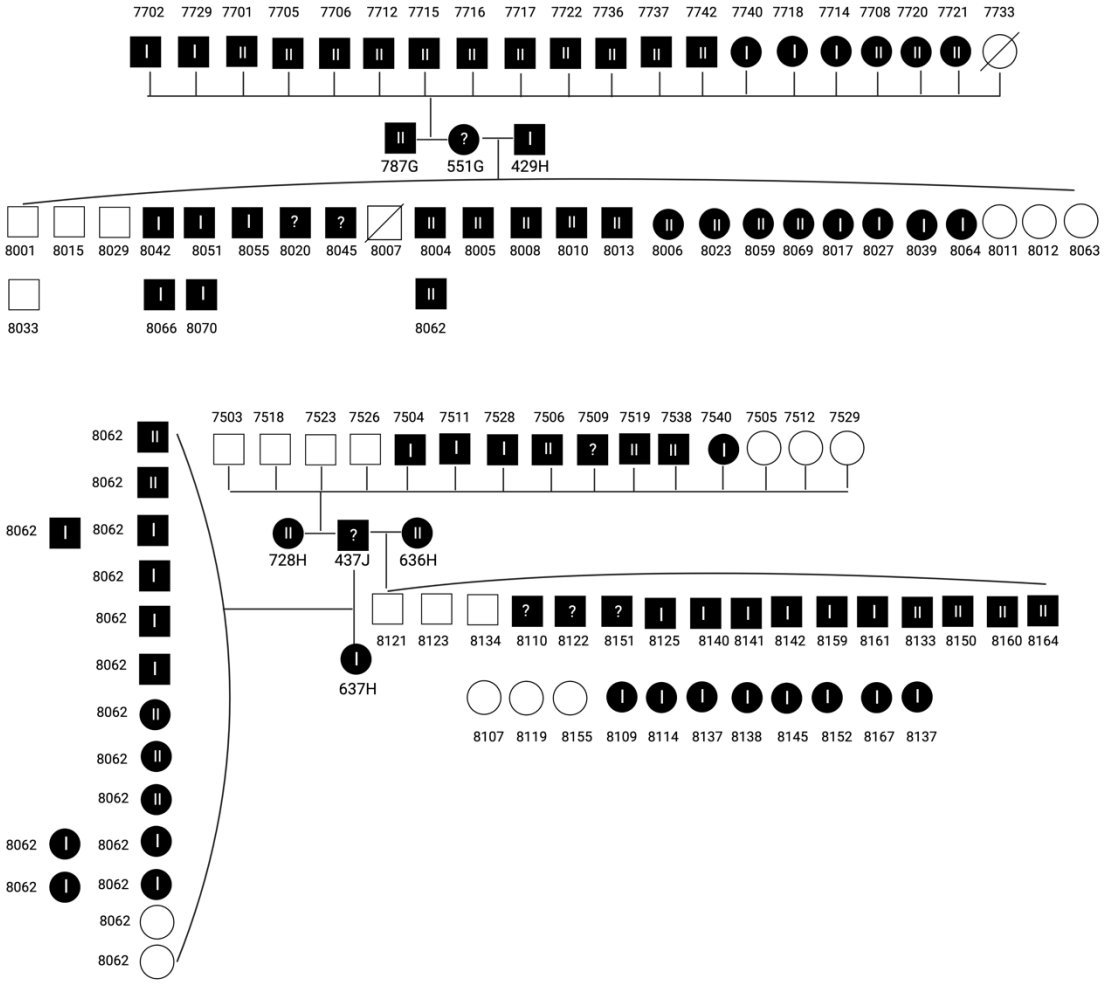
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8033 8066 8070 8062
7503 7518 7523 7526 7504 7511 7528 7506 7509 7519 7538 7540 7505 7512 7529
728H 437J 636H
8121 8123 8134 8110 8122 8151 8125 8140 8141 8142 8159 8161 8133 8150 8160 8164
637H
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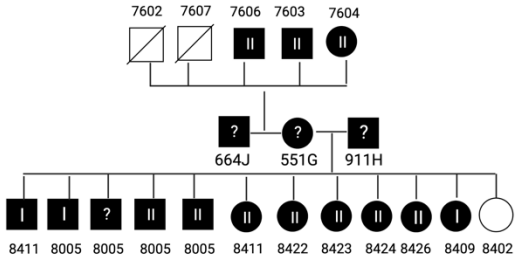
8062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7607
7606
7603
7604
?
?
?
664J
551G
911H
I
I
?
II
II
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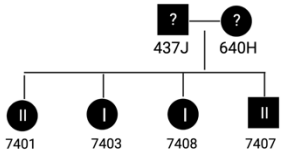
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I
II
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7403
7408
7407

APPENDIX B

COMPARISON OF SNP MARKER LIFT OVER WITH A PREVIOUS LIFT OVER TO ARS-UCD1.2 ASSEMBLY

Chromosome	Match	Number of markers	Proportion
	Perfect[1]	23057	0.5024
	Little[2]	22782	0.4964
1	Largely[3]	52	0.0011
	Perfect	19862	0.5025
	Little	19631	0.4967
2	Largely	31	0.0008
	Perfect	17605	0.5014
	Little	17472	0.4977
3	Largely	32	0.0009
	Perfect	16996	0.4948
	Little	17326	0.5044
4	Largely	29	0.0008
	Perfect	17081	0.4966
	Little	17278	0.5023
5	Largely	38	0.0011
	Perfect	17557	0.5022
6	Little	17380	0.4971

Chromosome	Match	Number of markers	Proportion
	Largely	24	0.0007
	Perfect	16248	0.4966
	Little	16449	0.5027
7	Largely	23	0.0007
	Perfect	16713	0.4964
	Little	16919	0.5026
8	Largely	34	0.0010
	Perfect	15103	0.4929
	Little	15510	0.5062
9	Largely	30	0.0010
	Perfect	14860	0.4982
	Little	14941	0.5009
10	Largely	25	0.0008
	Perfect	15822	0.4998
	Little	15817	0.4996
11	Largely	19	0.0006
	Perfect	12836	0.5007
	Little	12776	0.4983
12	Largely	25	0.0010
	Perfect	11518	0.4951
13	Little	11727	0.5041

Chromosome	Match	Number of markers	Proportion
	Largely	19	0.0008
	Perfect	12270	0.5019
	Little	12159	0.4974
14	Largely	16	0.0007
	Perfect	12028	0.4958
	Little	12210	0.5033
15	Largely	24	0.0010
	Perfect	11823	0.4966
	Little	11961	0.5024
16	Largely	22	0.0009
	Perfect	10873	0.4967
	Little	11002	0.5026
17	Largely	15	0.0007
	Perfect	9624	0.5043
	Little	9442	0.4948
18	Largely	18	0.0009
	Perfect	9301	0.4982
	Little	9352	0.5009
19	Largely	16	0.0009
	Perfect	10581	0.5004
20	Little	10550	0.4989

Chromosome	Match	Number of markers	Proportion
	Largely	16	0.0008
	Perfect	10232	0.4981
	Little	10290	0.5010
21	Largely	18	0.0009
	Perfect	8828	0.4961
	Little	8946	0.5028
22	Largely	20	0.0011
	Perfect	7507	0.4968
	Little	7588	0.5022
23	Largely	15	0.0010
	Perfect	9067	0.4921
	Little	9340	0.5069
24	Largely	19	0.0010
	Perfect	6343	0.4971
	Little	6412	0.5025
25	Largely	6	0.0005
	Perfect	7417	0.5016
	Little	7362	0.4978
26	Largely	9	0.0006
	Perfect	6581	0.4973
27	Little	6640	0.5017

Chromosome	Match	Number of markers	Proportion
	Largely	13	0.0010
	Perfect	6404	0.5005
	Little	6385	0.4990
28	Largely	7	0.0005
	Perfect	7226	0.5023
	Little	7144	0.4966
29	Largely	16	0.0011

[1]Perfect match comparing with coordinates obtained from Schnabel (2019).
[2]Coordinates are less than or equal to 2 bp from the coordinates obtained by Schnabel (2019).
[3]Coordinates are more than 2 bp from the coordinates obtained by Schnabel (2019).

APPENDIX C

SPATIAL EXPRESSION OF GENES OF INTEREST

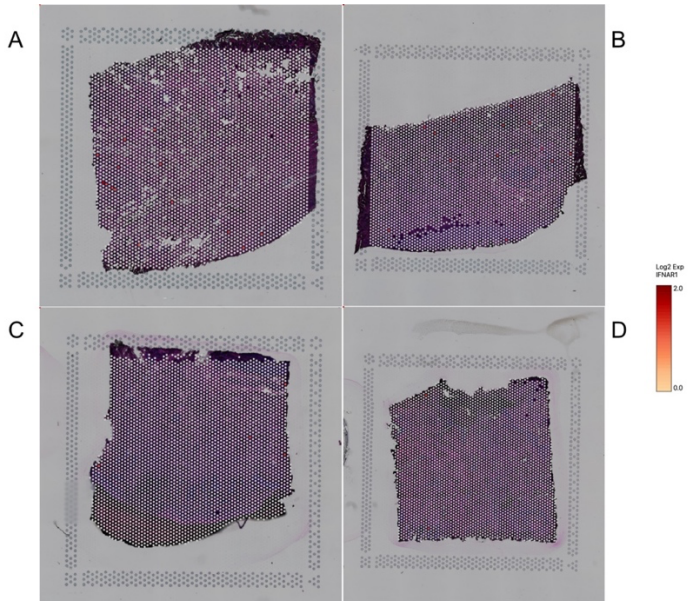

Figure S1 Spatial expression of *IFNAR1*. Layout is the same as in Figure 25.

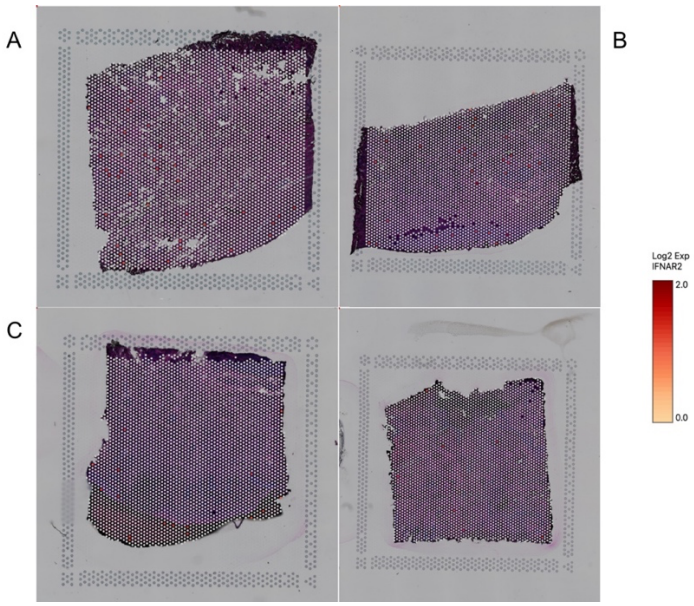

Figure S2 Spatial expression of *IFNAR2*. Layout is the same as in Figure 25.

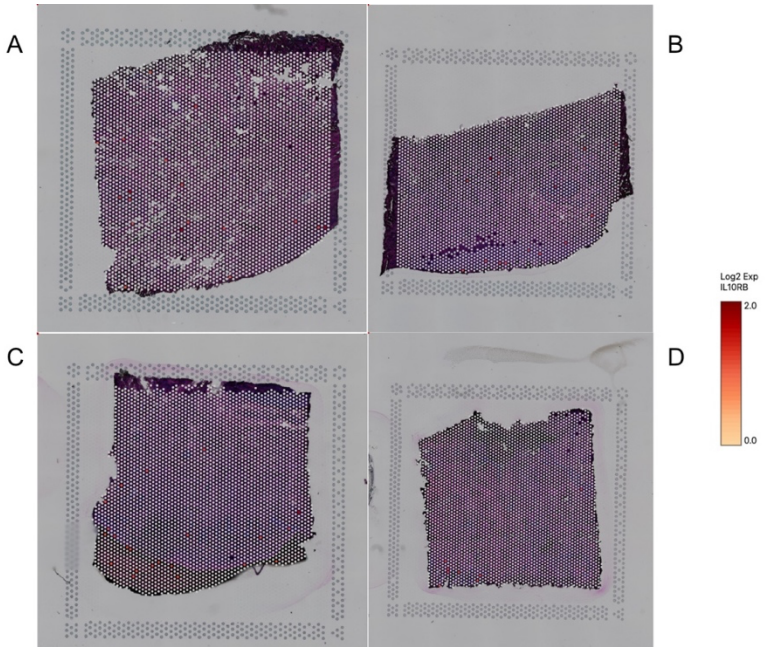

Figure S3 Spatial expression of *IL10RB*. Layout is the same as in Figure 25.

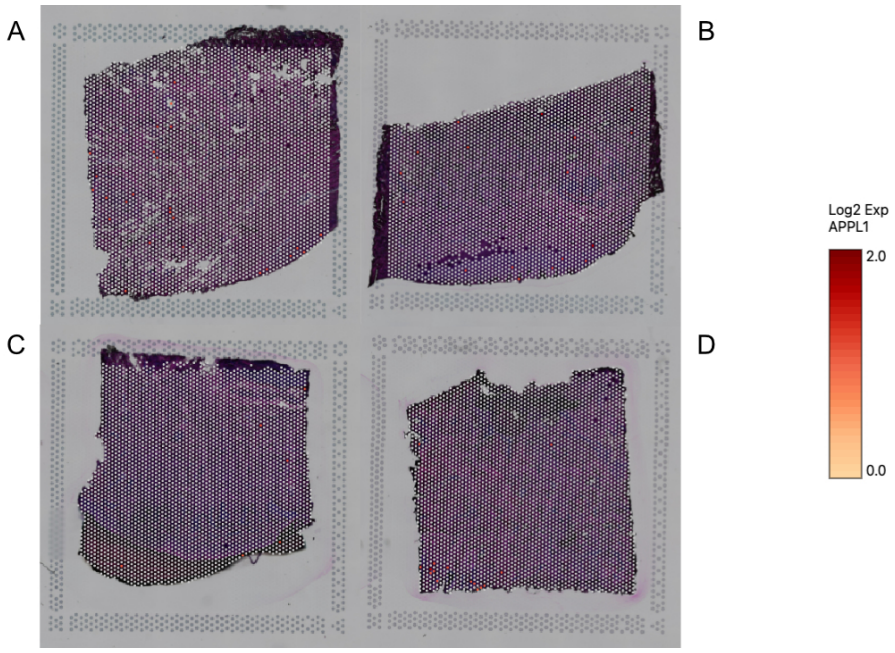

Figure S4 Spatial expression of *APPL1*. Layout is the same as in Figure 25.