Investigation of variation in genes influencing fertility in New Zealand sheep

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A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Doctor of Philosophy

at

Lincoln University

by

Hamed Amirpour Najafabadi

Lincoln University

2019

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An important trait in commercial sheep breeding is the number of lambs born per ewe, because the amount of meat produced per ewe is to a great extent determined by litter-size. Accordingly, the Identification of functional variation in genes that are responsible for improving fertility, would potentially allow for flocks to be bred for increased fertility, and thus increase profitability in the NZ sheep industry. Fertility if realised as increased fecundity, would not only be a determinant of profitability but may also affect the carbon footprint of New Zealand livestock production systems.

In this research, three genes involved in regulating fertility in sheep were investigated. Polymerase Chain Reaction – Single Strand Conformational Polymorphism (PCR-SSCP) analyses were used to search for genetic variation in three genes, the Growth Differentiation Factor 9 gene (*GDF9*), the Bone Morphogenetic Protein 15 gene (*BMP15*), and the Bone Morphogenetic Protein Receptor Type 1B gene (*BMPR1B*). Once identified by PCR-SSCP the genetic variation was further characterised with DNA sequencing. Confirmation of the sequence variation, then enabled subsequent testing of whether the variation was associated with variation in fertility in three sheep breeds (Finnish Landrace, Finnish Landrace X Texel and composites) using best linear unbiased prediction (BLUP) and ASREML with both animal and sire models.

In this study, the number of sheep studied was 1064 for the GDF9 gene and 852 for the BMP15 gene. A total of 241, 251 and 335 ewes were analysed for *GDF9, BMP15* and *BMPR1B* respectively. These included NZ Finnish Landrace sheep, Finnish Landrace × Texel-cross sheep, and composite sheep (farm 1) (of varying breed background). These three breeds derived from a single large ewe flock farmed on pasture and all fed in the same way in North Canterbury. All ewes had records for the 2016 lambing season, hence the number of lambs born in 2016 were used for association study.

In the sheep studied, variations in ovine *GDF9* and *BMP15* were associated with litter-size. Finnish Landrace × Texel-cross sheep with the c.1111A variant of *GDF9* were found to be more fertile (P = 0.036) than those without c.1111A. In animal models, the effect of *GDF9* appeared to be additive, with one copy of c.1111A increasing litter-size by 0.43 ± 0.202 in the Finnish Landrace x Texel-cross ewes, and two copies increasing litter-size by 0.86. No such effect was seen Finnish Landrace and composite sheep. However, the impact of a single copy of c.1111A led to an increase in litter-size of 0.34 ± 0.154 (p = 0.027) compared to those ewes with c.1111G, when all the sheep groups were analysed together. In contrast to the c.1111A>G results, litter-size did not differ between sheep with and without *GDF9* c.994A in all three groups of sheep investigated.

The c.31_33del in *BMP15* was found to be associated with litter-size (P < 0.001) in composite sheep. The effect of the presence of one copy of c.31_33del was an increase of 0.26 ± 0.092 (P = 0.008) lambs compared to those ewes without c.31_33del using the animal model. The estimate for the effect of variant A (absence of the c.31-33del) in the composite sheep was -0.26 ± 0.092 (p = 0.008) and -0.22 ± 0.095 (p = 0.026) in both the animal and sire models, respectively. This association between the detected c.31-33del and litter-size was not observed for Finnish Landrace or the Finnish Landrace x Texel-cross (P > 0.05). It is possible that the effect of this deletion in the signal sequence seems to vary from study to study and breed to breed.

Sequence analysis of a 394 bp fragment spanning the partial exon 9 and intron 8 and a 338 bp of exon 8 and intron 7 regions of *BMPR1B* in 335 sheep belonging to three groups of New Zealand sheep of differing background, revealed 5 variant sequences with a total of six single-nucleotide substitutions. The sequencing results revealed nucleotide substitutions c.1032T>C in the amplified region of exon 9/intron 8 and c.754-144G>A, c.754-88G>A, c.762G>A, c.754-31C>T and c.765G>A in the amplified region of exon 8/intron 7. Despite the presence of six nucleotide substitutions (found across two regions) in *BMPR1B*, no association was found between the sequence variation and litter-size (p > 0.05). This gene may not play a significant role in the fertility of the New Zealand sheep breeds investigated. The only modest (but not statistically significant, p = 0.162) association of intron 7/exon 8 was the effect of variant C on increased litter-size in composite sheep (0.23 ± 0.167). The impact of variant *B* in Finnish Landrace sheep (-0.04 ± 0.239 lambs, P = 0.861) is very similar to the effect of the variant *B* when all the groups were analysed together -0.04 ± 0.146 (P = 0.747).

The identification of functional sequence variation in the breeds studied here, may at first be of limited value to breeds that do not have the observed variation, but it lays a strong foundation to further this type of analysis with more common New Zealand breeds.

Keywords: Fertility, Litter-size, Growth differentiation factor (*GDF9*), Bone Morphogenetic Protein 15 (*BMP15*), Bone Morphogenetic Protein Receptor Type 1B (*BMPR1B*), NZ Sheep, Ovis aries, genetic selection.

Publications and Conference presentations arising from this thesis

Papers

Amirpour Najafabadi, H., Hickford, J.G.H., Zhou, H., & Khansefid, M. 2020. The effects of Single Nucleotide Polymorphisms in Growth Differentiation Growth Factor 9 Gene (*GDF9*) on Litter-size in three common New Zealand Sheep (*Ovis aries*) breeds. "Reproduction in Domestic Animals, accepted."

Amirpour Najafabadi, H., Hickford, J.G.H., Zhou, H., Z., Byun, S.O., & Fang, F. 2019. Genetic variation in the growth differentiation factor 9 (*GDF9*) gene in different New Zealand sheep (*Ovis aries*) breeds. "In preparation".

Conferences

Amirpour Najafabadi, H., Hickford, J.G.H., Zhou, H, & M. Khansefid, M. 2019. Identification of variation in the growth differentiation growth factor 9 (GDF9) gene associated with litter-size in New Zealand sheep (*Ovis aries*) breeds. 37th International Society for Animal Genetics Conference, Poster session- Applied Sheep and Goat Genetics.

Amirpour Najafabadi, H., Hickford, J.G.H., & Zhou, H. 2018. Having better genetic control over fertility in New Zealand maternal Sheep breeds. Proceedings of the World Congress on Genetics Applied to Livestock Production, Electronic Poster Session - Biology - Reproduction 2.11.801

Nucleotide Sequences Submitted to the NCBI GenBank

Ovine GDF9

Ovine growth differentiation factor 9 (GDF9) gene, GDF9 variant 1, A sequence: MK675521

Ovine growth differentiation factor 9 (GDF9) gene, GDF9 variant 2, B sequence: MK675522

Ovine growth differentiation factor 9 (GDF9) gene, GDF9 variant 3, C sequence: MK675523

Ovine BMP15

Ovine bone morphogenetic protein 15, (BMP15) gene, variant 1, *A* sequence: MN607693

Ovine bone morphogenetic protein 15, (BMP15) gene, variant 1, *B* sequence: MN607694

Ovine BMPR1B

Ovine bone morphogenetic protein receptor type 1B (BMPR1B) gene, exon 8, *A* sequence: MN607695

Ovine bone morphogenetic protein receptor type 1B (BMPR1B) gene, exon 8, *B* sequence: MN607696

Ovine bone morphogenetic protein receptor type 1B (BMPR1B) gene, exon 8, *C* sequence: MN607699

Ovine bone morphogenetic protein receptor type 1B (BMPR1B) gene, exon 9, *A* sequence: MN607697

Ovine bone morphogenetic protein receptor type 1B (BMPR1B) gene, exon 9, *B* sequence: MN607698

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisor Professor Jon Hickford for the continuous support of my Ph.D. study and related research for his patience, motivation, and immense knowledge. His heaps of advice show me the direction to write up this thesis. I would never have imagined that having a better advisor and mentor for my Ph.D. study. All my success and knowledge, I owe to him. I am grateful for his support!

Also, I would like to thank the rest of my thesis committee: Dr Huitong Zhou, for his insightful comments and encouragement, but also for the hard question which incentivised me to widen my research from various perspectives. I am particularly grateful to Dr Majid Khansefid for his valuable advice and help, and most of all, his good nature that goes with it.

My sincere thanks also go to Andrew Hogan and Freeman Fang, who gave access to the laboratory and research facilities. Also, I thank my friend in the Bio-Protection Research Centre (Dr Hossein Alizadeh) for his precious support.

I would also like to acknowledge the sources of funding for this work. New Zealand International Doctoral Research Scholarship (NZIDRS), Kathleen Ann Stevens Scholarship and Macmillan Brown Agricultural Research Scholarship. Thank you to Christine Roberts, Anna Dekkers, and Jane Edwards for helping with scholarships. Thank you again for your generosity and support. Your generosity has inspired me to help others and give back to the community. I hope one day I will be able to help students achieve their goals just as you have helped me.

I particularly wish to thank Jenny Juengel of AgResearch, for her advice throughout my studies. Thanks also to the AGLS group for their friendliness and helpfulness; Craig Bunt, Robyn Wilson, Alison Hind. I also thank Mr Daniel Wheeler for his collaboration in providing blood samples, data, and pedigree for my experimental work.

I thank my fellow lab-mates for the stimulating discussions, and for all the fun we have had in the last four years. Also, I appreciate my friends in the following institution. I am also grateful to my friends (Ghassan, Kelly, Sali, Shayan, Babak, Richard, Jan, Grace, Léonie, Leo, Reza, Leila, Ishaku, Jane, Lucy, Ivy) and I hope these new and old friendships will last a lifetime. I just wanted to write to let you know how much I appreciate the positive influence you've had on my life. Thanks for all you do and useful advice! I will forever be grateful.

Lastly, and most significantly, I would like to offer my sincere appreciation to my lovely parents (Rouholamin and Zahra), my brothers and their wives for their affection, moral support and encouragement to complete this thesis. I would like to dedicate my thesis to my parents, without whose unwavering support it could not have been completed. All their help was invaluable throughout my Ph.D. To my mom and dad, Thank you for always being my side. A life without them is like a flower without petals; I don't know where I would be. Thanks for walking ahead of me. And I love you both more than anything in my life. I promise I will make you proud.

Table of Contents

List of Tables

List of Figures

Abbreviations

µg	microgram	EDTA	Ethylenediaminetetraacetic acid
M	Molar	FTA	Flinders Technology Associates
µL	microlitre	TE	Tis-EDTA buffer
µm	micrometre	SIL	Sheep Improvement Limited
mg	milligram	HCOH	hydroxymethylene
µM	micromolar	NAOH	Sodium hydroxide
mL	Millilitre	Kbp	Kilo base pair
ng/mL	nanograms per millilitre	NCBI	National Centre for Biology Information
A	adenine	PCR	Polymerase Chain Reaction
C	cytosine	SNP	Single nucleotide polymorphism
T	thymine	RFLP	Restriction Fragment Length Polymorphism
G	guanine	cDNA	complementary DNA
aa	amino acid	Taq	*Thermus aquaticus*
nt	nucleotide	TBE	tris-borate-EDTA
bp	base pair	dNTP	deoxyribonucleoside
h	hour	EDTA	ethylene diamine tetra acetic acid
PH	Potential of Hydrogen	GDF9	Growth Differentiation Factor 9
V	volt	BMP15	Bone morphogenetic protein 15
NZ	New Zealand	BMPR1B	Bone morphogenetic protein Receptor Type 1B
U	Unit	ALK6	activin-like kinase 6
TE	tris-EDTA	TGF ß superfamily	transforming growth factor beta (TGF-β) family
oC	degree celcius	Reml	Residual Maximum Likelihood
		Tris	tris (hydroxylethyl) aminomethane
		SSCP	Single-Strand Conformation Polymorphism

Chapter 1

Introduction and Literature Review

1.1 Introduction

It is projected by the United Nations (UN) that the global population will increase to 9.7 billion people by 2050 (United Nations, 2019). If the consumption of livestock products per person does not change, then it is expected that at least 1.5 times more product than currently produced will be required (FAO, 2003).

Sheep are one of the more common livestock species farmed. They are distributed across the globe, being found equatorially and at higher latitudes too. Their ability to live in different environments, including a wide range of temperatures, rainfalls and altitudes, is in part attributable to the ability to maintain reproductive performance. What-is-more, as ruminants they can survive on a diversity of forage sources and for extended periods of time with very low feed intakes. They also have the ability to go in search of food and they can cover large distance in that quest.

Sheep have been selected to be multi-purpose animals for production. They can produce fibre (wool), meat, milk and skins. To achieve this production, the most important aim of sheep breeding is to maintain a high reproductive rate to sustain replacement flocks for both milk and meat production. In New Zealand specifically, as a major lamb exporter, the primary focus is to produce lambs for slaughter. Higher rates of fertility are therefore very desirable, provided the farming system can support that reproductive performance.

Animal breeding is one of the ways to improve animal productivity and meet the above demand. It can increase the quantity and quality of production, but it can be a very slow process. Breeding programs are therefore usually considered to be a long-term approach to increasing livestock production (Henryon et al, 2014). A well designed breeding program will attain a breeding goal or objective through the selection of the best animals for achieving that goal (Flint & Woolliams, 2007), and optimised breeding strategies can lead to genetic gain and prevent inbreeding. Van der Werf (2007) describes how estimated breeding values (EBVs) have been calculated for many traits of economic importance, and how they can be used to improve the accuracy of animal evaluation and selection in any given breeding program.

Making genetic gain in key livestock traits like reproductive performance is typically very slow, and both the fecundity and fertility of the livestock need to be considered. Fecundity means the 'ability' to produce live offspring, while fertility means the 'actual production' of live offspring (i.e. fecundity refers to the potential for production, and fertility is the actual production of live offspring). In livestock production, while male fecundity is important, non-performing males can usually be rapidly detected and culled. Given that it is the female that produces the progeny that will become the next generation, the ability to accurately assess a female's reproductive performance, especially fertility, can only really be undertaken at the end of her reproductive life. This makes breeding for increased fertility rather challenging.

In New Zealand (NZ), genetic selection for different production traits in sheep has been undertaken using a genetic evaluation system called Sheep Improvement Limited (SIL; https://www.sil.co.nz/). This system calculates EBVs for various production traits, including EBVs for the number of lambs born (NLB, or litter-size), growth and weight performance, and carcass traits that are of value. In the context of fertility, it has led to gain in the number of lambs born (Beef+Lamb New Zealand Economic Service., 2016).

This reproductive success is measured at a national level as lambing percentage, this being a measure of the number of lambs produced, per ewe mated. During the period 1935-2011, there has been marked improvement in this performance measure in New Zealand, with an increase from 0.8-0.9 lambs per ewe (80-90%) to approximately 1.02 (102%) and 1.16 (116%) in 1989 and 2011, respectively Figure 1 (Stats NZ, 2017).

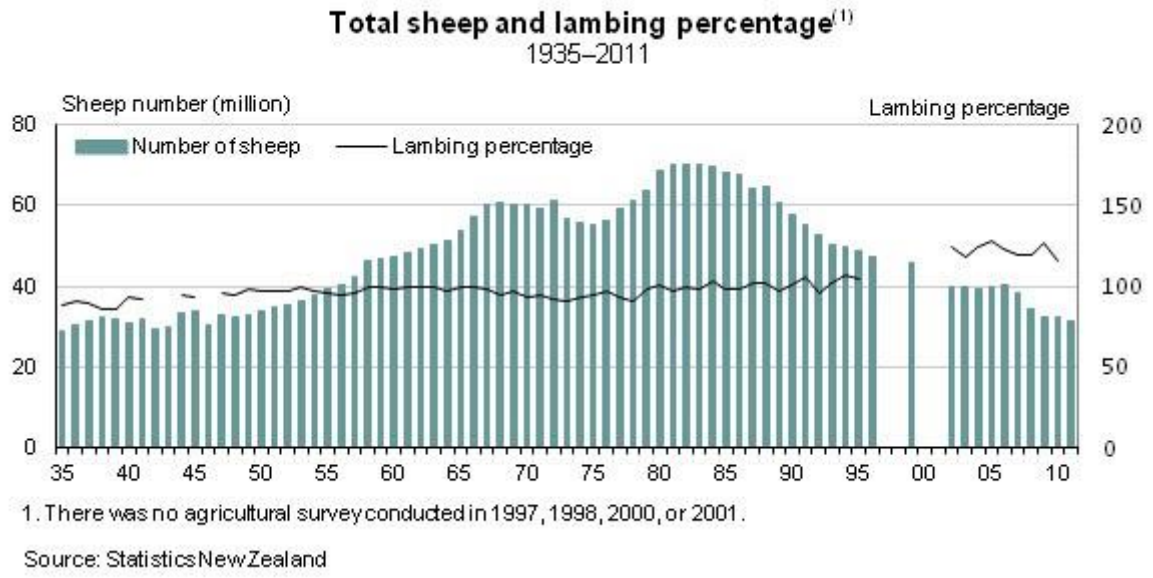


Figure 1 Total number of sheep and lambing percentage between 1935 and 2011 in New Zealand (Stats NZ, 2017)

More recently Beef+Lamb NZ (Beef+Lamb New Zealand Economic Service., 2016), has described the effect in more detail, the decline in lambs produced in NZ being somewhat less marked than the reduction in breeding ewe numbers (Figure 2).

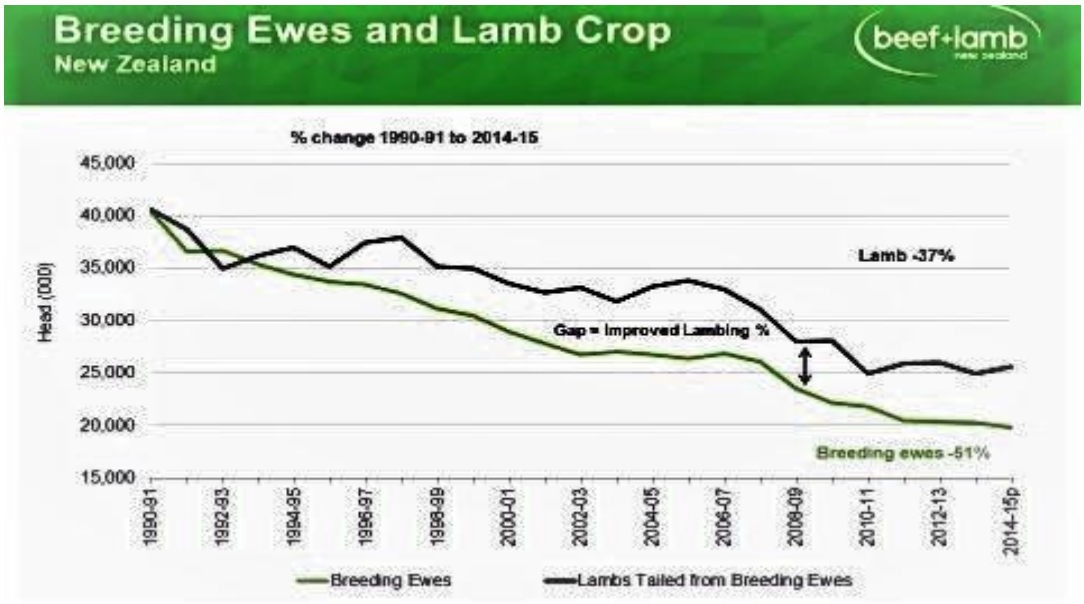


Figure 2 Trends in New Zealand breeding ewe and lamb numbers over the 1990s and early 2000 decades (Source: Beef+Lamb New Zealand economic service, 2016)

There is evidence that improvements in genetic evaluation and breeding can be achieved by having a better understanding of both the genome and individual genes in livestock species, and typically the use of DNA information enables us to increase the rate of genetic gain compared to using only phenotypic information (Meuwissen et al., 2001). Research has identified useful information about sheep genetics and the use of 'DNA markers' to improve performance is becoming widespread. Specifically, animals with better performance for key production traits of economic significance, can be selected for use as breeding stock by both commercial farmers and sheep breeders.

Previous research has established that variation between individual sheep in reproductive performance, can be the result of variation in both their genetics and the environment in which they are farmed. There is also likely to be genotype by environment, or non-additive combination effects (Lush & Mollin, 1942). This provides a context to search for genes and genetic variation that affects reproductive performance in sheep.

1.2 Why is fertility important in sheep production?

Fertility is widely considered to be the most important trait as regards sheep productivity, with the number of offspring obtained per lambing (variously described as 'litter-size', or 'number of lambs born') being a useful indicator of fertility rates. According to some authors (Petrovic, 2000) fertility is more important than production in lambs (i.e. their biological capacity for meat, milk and wool production), because these factors are ultimately affected by fertility (Notter, 2008).

Equally, various studies have described how perinatal mortality results in major economic losses to the sheep industry (Amer, 2000; Dalton et al., 1980; Darby et al., 1992; Sykes & Dingwall, 1976). It has therefore been suggested that the weight of meat and wool produced each year is more dependent upon the total number of lambs that survive to weaning, than upon the individual performance of the lambs (Lax & Newton, 1965; Sidwell et al., 1962). It could also therefore be argued that the selection for enhanced lamb survival is economically more important than selection for production traits. Taken together this explains the challenge that can exist between increasing fertility and thus increasing the number of lambs born, versus the survival of those lambs, especially if the lambs are of a markedly reduced or elevated birth weight, and thus more susceptible to perinatal mortality (Dalton, 1979).

1.3 Factors that affect fertility

Prior to discussing the genetic factors that might affect fertility, it is important to briefly touch on the environmental factors that can affect litter-size. These include factors that can be managed in farming systems, such as nutrition, but also less manageable environmental effects such as climate.

1.3.1 Environmental and management factors that affect fertility

A variety of environmental factors can affect sheep reproductive performance in sheep. For example, heat stress reduces performance (Hansen, 2009), with high summer temperatures affecting semen quality and reducing sexual activity (Petrović et al., 2002).

The importance of farm management in fertility has been described by several researchers (Anel et al., 2005; Paulenz et al., 2002), and there are numerous reviews detailing both general and very specific detail on the effect of nutrition on fertility. For example, Robinson et al. (2006) described the nutritionally sensitive affects fertility indirectly through its impact on the circulating concentrations of the hormones and other nutrient-sensitive metabolites periods in the production of gametes and viable embryos, and provided a conceptual framework from which to develop long-term feeding strategies that enable sheep fertility to be maximised. At a more pragmatic level in New Zealand,

Beef+Lamb NZ has scientifically-based resource material describing how to optimise feeding systems for sheep farmers (New Zealand Sheep Council, 1994).

Although environmental and management factors are important factors affecting sheep fertility, only genetic factors are permanent and can be passed to the next generation through sheep breeding. They are therefore the primary focus of this literature review.

1.3.2 Genetic factors that affect fertility

Advances in selection for increased fertility depend on the genetic variability of key reproductive components (Petrović et al., 2007; Petrović et al., 2002; Petrović et al., 1997; Petrović et al. , 2001). However, heritability estimates for fertility traits are typically low and they have been reported to vary between 0.1 and 0.26 (Petrović, 2000). This suggests a complex genetic background underpins the traits. It has however been argued that genetic improvement in litter-size can be achieved by three main methods:

1) The use of breed resources of differing reproductive capability, 2) selection within a given breed for superior individuals, and 3) the use of technologies that enable major genes to be selected for (Elsen et al., 1994).

The combination of low heritability estimates for fertility, discrete phenotypic expression and realisation of fertility only being easily measured in sexually mature ewes, does however lead to typically low selection intensities and long generation intervals in breeding for fertility. This has driven the search for major genes that influence fertility traits.

From this perspective, understanding genes that underpin variation in ovulation rate has become important. Ovulation is the release of an oocyte from the ovary, and it is the culmination of an integrated and synchronised succession of hormonal actions and morphological changes that principally involve the anterior hypothalamus, the anterior pituitary, and the ovaries themselves. The major protein 'players' in this system are gonadotropin releasing-hormone (GnRH), follicle stimulating hormone (FSH), luteinising hormone (LH), oestrogen and progesterone, but fine-tuning of this system is provided by a many other factors including inhibin, activin, and other growth factors. Accordingly, the specific genes that produce the proteins involved in these processes can be key determinants of fertility, and this will be addressed in more detail below.

Genetic variation in ovulation rate in sheep has been studied in different breeds, and it is now understood that prolificacy can be affected by the segregation of major genes associated with reproduction and ovulation (Mulsant et al., 2001; Wilson et al., 2001). What-is-more, in the past two

decades, geneticists have located some of these genes on chromosomes, described their nucleotide sequence and ultimately described the nucleotide sequence variation that affects ovulation.

1.4 Genes that affect fertility in sheep

The identification of genes that affect ovulation rate and other reproductive traits, is now allowing more rapid progress in breeding sheep for increased fertility. Genes affecting the rate of synthesis and function of gonadotropins, uterine size, etc., have been identified.

Key genes that affect sheep fertility have been described (Davis et al. , 2001; Demars et al., 2013; Mullen & Hanrahan, 2014; Nicol et al., 2009; Souza et al., 2001; Våge et al. , 2013; Wilson et al., 2001). These include Bone Morphogenetic Protein Receptor Type 1B (*BMPR1B*) (also known as *Alk6, SKR6, ALK-6, AMDD, BDA2, BDA1D, CDw293, Acvrlk6, BMPR-1B, BMPR-IB, CFK-43a, AI385617* and *AV355320*), Growth Differentiation Factor 9 (*GDF9*) (also known as *GDF-9, POF14*), Bone morphogenetic protein 15 (*BMP15*) (also known as *GDF9B, BMP-15, GDF-9B, ODG2 and POF4*), Beta-1,4 N-acetylgalactosaminyltransferase 2 (*B4GALNT2*) (also known as *B4GALT* and *GALGT2*), Wishart (*FecW*) and *FecX2*, a yet to be identified X- linked mutation (Davis et al., 2001, 2006), which has been found in Woodlands (W) ewes (*FecX2W*). Both the heterozygous and homozygous carrier animals for Woodlands have higher ovulation rates and litter- sizes (Davis, 2005; Davis et al., 2001)

The homeobox protein prophet of Pit-1 (*PROP1*) gene (*PROP1*, also known as *CPHD2, PROP-1,* and *PROP paired-like homeobox 1*) also plays a vital role in fertility. There are twelve reported mutations in the human *PROP1* that may prevent the production of several hormones leading to either absence or delay of secondary sexual development and infertility (Navardauskaite et al., 2014; Sornson et al., 1996; Taha, Mullis, Ibáñez, & De Zegher, 2005). Some of the above mentioned mutations in humans have been uncovered in sheep, most notable of which is a C>T transversion at position 330 (ENSOART00000007395: c.109+207C>T) in intron 1, and this is a potential molecular marker to improve litter-size of sheep (Liu et al., 2015).

Of the genes described above, three were chosen for further analysis in this study. They were *BMPR1B*, *GDF9* and *BMP15*.

1.5 The genes that were studied in this thesis

The proteins GDF9 and BMP15 belong to the transforming growth factor-β (TGF β) family, a large group of structurally related proteins that regulate the expression and secretion of hormones that affect follicular growth and ovulation rates. Members of the TGF-superfamily share several characteristics and the biologically active (i.e., mature) regions of most of these proteins are usually quite small. Of the TGF-superfamily, both GDF9 and BMP15 are produced as precursor proteins, with

the biologically active portion of the protein residing in the c-terminus (Juengel et al., 2004). The precursor proteins are 453 and 393 amino acids in length for ovine GDF9 and BMP15 respectively, and they consist of a short secretory signal sequence followed by a pro-region with the final 135 (oGDF9) or 125 (oBMP15) amino acid sequences comprising the mature or biologically active regions of the proteins (Bodensteiner et al., 1999; Galloway et al., 2000). Although the function of the pro-region portions of GDF9 and BMP15 is unknown, in other TGF-family members this is thought to facilitate the correct folding and dimerisation of the mature proteins, and hence they may be necessary for regulating biological activity (Barker, 1994; Chang et al., 2002). The mature regions can dimerise with themselves (to form homodimers) or with the mature regions of other TGF-superfamily members (to form heterodimers) (Mottershead et al., 2015).

The mature regions of most TGF-superfamily members contain an odd number of cysteine residues (typically seven), with six of these residues forming a characteristic cysteine knot, and the remaining cysteine involved in creating a disulphide bond between the two mature regions (Chang et al., 2002). The GDF9 and BMP15 proteins are however two of the very few TGF-superfamily members that do not have the cysteine residue that is involved in dimer formation. As such, it is unclear if the structure of dimers is a necessary prerequisite for their biological activity (as it is for other TGF-superfamily members). Moreover, as both proteins are produced in the oocyte, the potential for production of biologically active heterodimers of GDF9/BMP15 certainly exists. Recently, it was shown that both heterodimers and homodimers of GDF9 and BMP15 could be formed when produced in transfected cell lines (Liao et al., 2003), but the biological activity of these dimer proteins was not tested.

There is a large body of literature describing the activity of GDF9 and BMP15 in cows, sheep, and pigs, and how variation in these genes affects reproductive performance. It has also been reported that variation in these genes can be used as a marker to increase litter-size and ovulation rate in mammals. This will be discussed in more detail below.

1.5.1 Growth Differentiation Factor 9 and the GDF9 gene (*GDF9*, Ensembl: ENSOARG00000013229.1, also known as *GDF-9*, *POF14*)

Growth differentiation factor 9 (GDF9) is a protein that is secreted in mammals by growing follicular oocytes (McPherron & Lee, 1993). The gene is expressed in the oocytes and is essential for follicle production (Bodensteiner et al., 1999), granulosa cell growth (Davis, 2005), the rate of oocyte maturation, premature ovarian activity (Galloway et al., 2000), and in the differentiation and maturation of oocytes. It is now well established from a variety of studies that GDF9 is necessary to produce ovarian follicles in sheep (Hanrahan et al., 2004) and the importance of regulating the

process of folliculogenesis by GDF9 is illustrated by the observation that an absence of this factor leads to the cessation of follicular growth and development (Chang et al., 2002).

The GDF9 gene (*GDF9*) is expressed in oocytes from the primary stage of follicular development until ovulation (Laitinen et al., 1998; McGrath et al. , 1995) and female *GDF9* knockout mice (*GDF9-/-*) are infertile due to a block in follicular development at the primary stage (Dong et al., 1996). While the *GDF9-/-* female mice were sterile, the heterozygous females were fertile. In this knockout mouse model, the ovaries from female mice deficient in *GDF9* produced primordial and primary 1- layer follicles, but there was a block in follicular development beyond the primary 1-layer follicle stage, which led to complete infertility. Oocyte growth and zona pellucida formation proceeded normally, but other aspects of oocyte differentiation were compromised. The oocytes in the knockout mice grew faster and had more structural defects (Carabatsos et al., 1998). Additionally, the levels of FSH and LH were elevated, and ovarian cysts were often observed (Dong et al., 1996). Aberrant expression of mRNA encoding several proteins was observed in the mice lacking GDF9, with ovarian tissue expression of stem cell factor (SCF) being increased, whereas expression of aromatase, activin-B, follistatin, and COX-2 was decreased compared to the *GDF9*-intact controls (Dong et al., 1996; Elvin et al. , 1999). Changes in the above mentioned mRNAs appear to be a consequence of the block in follicular growth (and thus the absence of more mature follicles), along with the presence of abnormal nests of luteinizing granulosa cells following degeneration of the oocyte and the loss of an interactive feedback system.

Aaltonen et al. (1999) determined the localisation of the *GDF9* mRNA and protein during folliculogenesis in humans using in-situ hybridization and immuno-histochemical analyses, and compared it with that of a related protein growth differentiation factor 9B (GDF9B), which is now called bone morphogenetic protein 15 (BMP15 – see below). The *GDF9* transcripts were not detected in primordial follicles, but were abundantly expressed in primary follicles in frozen sections of ovarian cortical tissue. The human *GDF9B* transcripts could only be detected in the gonads by RT-PCR analysis, and *in-situ* hybridization studies indicated that *GDF9B* is not expressed in small primary follicles, but instead in the oocytes of the late primary follicles.

From the above work, Aaltonen et al. (1999) concluded that both *GDF9* mRNA and protein are abundantly expressed in oocytes of primary follicles in human ovaries, suggesting that the *GDF9* transcript is translated at this early stage of folliculogenesis; that BMP15 is specifically expressed in gonads at low levels; that the expression of *GDF9* mRNA begins slightly earlier than that of *BMP15* in human oocytes during follicular development; and that the results are consistent with the suggestion that GDF9 and BMP15 regulate human folliculogenesis in a manner specific to the ovary.

Filho et al. (2002) compared the pattern and level of expression of *GDF9* and *BMP15* mRNA in ovaries from normal-cycling individuals, with women with polycystic ovary syndrome (PCOS) and polycystic ovaries (PCO). *In-situ* hybridisation suggested that the expression of *GDF9* and *BMP15* was restricted to oocytes in all the ovaries examined, with a decreased level of *GDF9* signal observed in developing PCOS and PCO oocytes, compared with normal oocytes. This difference was evident throughout folliculogenesis. The results indicated that the expression of *GDF9* mRNA is delayed and reduced in PCOS and PCO oocytes during their growth and differentiation phase, and because oocyte- derived GDF9 is crucial for normal folliculogenesis and female fertility, Filho et al. (2002) suggested that a dysregulation of oocyte *GDF9* expression may contribute to the aberrant folliculogenesis in PCOS and PCO women.

Sheep *GDF9* has been mapped to sheep chromosome 5 (Sadighi et al., 2002). The gene spans about 2.5 kilobases (kb) and contains 2 exons separated by a single 1126-base pair (bp) intron and encodes a pre-propeptide of 453 amino acid residues. The active mature peptide is 135 amino acids long (Bodensteiner et al., 1999).

Genetic variation and mutations in the ovine GDF9 gene

Nucleotide sequence variation has been described for ovine *GDF9*. Some of this variation causes a loss of fertility and thus can be deemed to be a mutation, while other sequence variations are more benign, and have only minor effects. Some of the nucleotide substitutions that affect fertility are listed in Table 1, while a comprehensive description of nucleotide variation can be found at Ensembl (https://www.ensembl.org/Ovis_aries/Transcript/Variation_Transcript/Table?db=core;g=ENSOARG00000013229;r=5:41841034-41843517;t=ENSOART00000014382). Figure 3 is a graphical representation of the currently identified nucleotide sequence variation in the gene.

Table 1 Growth Differentiation Factor-9 gene (*GDF9*) nucleotide changes/mutations and their effects on ovulation rate and litter-size

Name	Nucleotide sequence change*	Reference SNPs	Amino acid change	Effect on ovulation rate and litter-size	Reference
	ENSOART00000014382.1: c.152A>G	-	p.Asn51Asp	Unknown	(Liao et al., 2003)
G1 (FecG1)	c.260G>A	rs410123449	p.Arg87His	Increased ovulation rate and the majority of the sterility phenotypes in these animals can be explained by the presence of homozygous mutant (His/His) genotypes.	(Hanrahan et al., 2004)
				Litter-size in (+/+) and (+/-) genotypes was equal to 1.16 ± 0.05 and 1.78 ± 0.05 respectively.	(Javanmard, Azadzadeh, & Esmailizadeh, 2011)
				Did not affect reproductive traits.	(Abdoli, Zamani, Deljou, & Rezvan, 2013)
				Litter-size in (+/+) and (+/-) genotypes was equal to 1.25 ± 0.09 and 1.56 ± 0.08 respectively.	(Paz, Quinones, Bravo, Montaldo, & Sepulveda, 2015)
				One copy of each of the *BMP15* and *GDF9* mutations had equivalent effects on ovulation rate in Moghani and Ghezel sheep.	(Barzegari et al., 2010)
G2	c.471C>T	rs422644056	p.157 - No change	No association was found with litter-size.	(Hanrahan et al., 2004) (Albarella et al., 2015)
G3	c.477G>A	rs160076413	p.159 - No change	No association was found with litter-size.	(Hanrahan et al., 2004) (Albarella et al., 2015)
	c.692T>C	-	p.Leu231Thr	Unknown	(Guan et al., 2005)
G4	c.721G>A	rs160076408	p.Glu241Lys	Increased ovulation rate and the majority of the sterility phenotypes in these animals can be explained by the presence of homozygous mutant (Lys/Lys) genotypes.	
	c.729G>T	-	p.Gln243His	Homozygous wild type and heterozygote had 2.11 ± 0.10 and 2.99 ± 0.19 lambs per litter respectively in Small Tail Han sheep.	(M. X. Chu, Li, Wang, Ye, & Fang, 2004)

	c.750G>A	rs193637058		The GG homozygous individuals displayed a significantly higher value of litter-size when compared with GA ewes in the Bagnolese and Lori breed	(Albarella et al., 2015) (Zamani, Abdoli, Deljou, & Rezvan, 2015)
FecG7	c.943C>T	-	p.Arg315Cys	Increased ovulation rate and litter-size in heterozygous and infertility in homozygous ewes.	(Souza, McNeilly, Benavides, Melo, & Moraes, 2014)
G5	c.978A>G	rs399579080	p.326 – No change	No association was found with litter-size.	(Hanrahan et al., 2004) (Albarella et al., 2015)
G6	c.994G>A	rs421019907	p.Val332Ile	The increased ovulation rate and the majority of the sterility phenotypes in these animals can be explained by the presence of heterozygous mutations and homozygous mutations, respectively.	(Hanrahan et al., 2004) (Albarella et al., 2015)
$FecG^{E}$/$FecG^{SI}$ Embrapa	c.1034T>G	rs1092755620	p.Phe345Cys	$FecG^{E}$ homozygous ewes are not sterile but show a significant increase compared to non-mutated individuals ovulation rate (2.22 ± 0.12 vs. 1.22 ± 0.11) and litter-size (1.78 vs. 1.13) in Brazilian Santa Ines sheep breed. The average number of corpora lutea in the homozygous ewes was more than heterozygote or wild type animals.	(Silva et al., 2011) (Melo et al., 2008)

[+]Nucleotide positions relative to GenBank AF078545.2

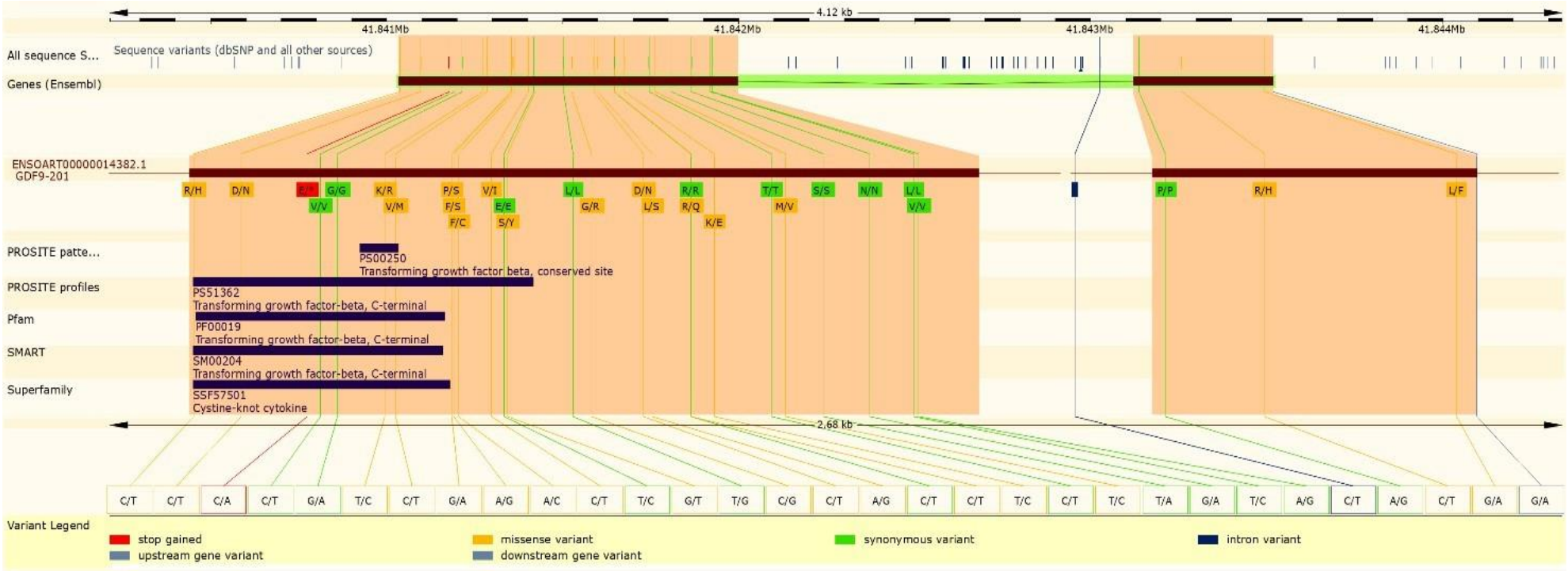


Figure 3 Ensembl image file of ovine *GDF9* sequence variation (https://www.ensembl.org/Ovis_aries/Transcript/Variation_Transcript/Table?db=core;g=ENSO ARG00000013229;r=5:41841034-41843517;t=ENSOART00000014382)

Over the last decade, the sheep industry has utilized *GDF9* marker-assisted selection (MAS) to identify fertile sheep and improve the quality of sheep breeding programs.

The economic impact of some of the variations identified in *GDF9* is very high. For example, the nucleotide sequence variation (c.1111G>A) identified in Cambridge and Belclare sheep, was strongly associated with litter-size in Norwegian White sheep (Våge et al., 2013). This nucleotide variation was also detected in the Finnish Landrace breed in NZ, and this has enabled the development of a commercial gene-marker for *GDF9* variation for use in improving fertility by the Gene-Marker Laboratory at Lincoln University, New Zealand.

The New Zealand Sheep Breeders Association reported that the presence of *GDF9* mutation has the potential to increase ovulation in Texel sheep by 25-40% (Gardyne, 2017).

The presence of *GDF9* c.1034T in the Brazilian Santa Ines and Morada Nova hair has been shown to increase litter-size, and this mutation is used as a commercial marker to improve sheep production in Brazil. The uncommon presence of the beneficial *GDF9* and Booroola mutations in Iranian sheep breeds (Eghbalsaied et al., 2017; Nanekarani et al., 2016) has led to the importation of higher fertility sheep carrying the functional mutations. The GDF9 gene has been used as a marker to increase fertility in different research stations around Iran (http://www.avingen.com).

1.5.2 Bone Morphogenetic Protein 15 and BMP15 gene (*BMP15*: Ensembl: ENSOARG00000009372, also known as *GDF9B, BMP-15, GDF-9B, ODG2* and *POF4*)

The bone morphogenetic protein (BMP) family is also part of the transforming growth factor-beta superfamily. These proteins are typically synthesised as pre-propeptides, cleaved, and then processed into dimeric proteins. With a few exceptions, members of the TGFB superfamily are defined by seven spatially-conserved cysteine residues (Dube et al., 1998). Using degenerative oligonucleotides to target the conserved amino acids of the BMP/Vg1/DPP subgroup of the TGFB superfamily, Dube et al. (1998) identified an additional member of the BMP family, BMP15 (also referred to as GDF9B), in both mouse and human. The pre-propeptides exhibit an amino acid identity of 63%, and both have five potential N- linked glycosylation sites, of which three are spatially conserved between the species (Dube et al., 1998).

Using Northern blot analysis, Dube et al. (1998) revealed that mouse *BMP15* is expressed only in the ovaries. *In-situ* hybridization revealed that murine *BMP15* was expressed exclusively in the oocyte soon after primordial follicles are recruited, and that expression is maintained until after ovulation. The spatio-temporal patterns of BMP15 and GDF9 activity are identical, such that Dube et al. (1998)

suggested that BMP15 may be involved in oocyte maturation and follicular development as a homo-dimer, or by forming hetero-dimers with GDF9.

Using *in-situ* hybridisation and immuno-histochemical analysis, (Otsuka et al., 2000) demonstrated selective and increasing expression of BMP15 in oocytes throughout follicular development. Immunoblot analysis detected 16- and 50-kD proteins. Functional analysis showed that the addition of BMP15 to rat granulosa cells increased proliferation and DNA synthesis, which was unaffected by FSH. The BMP15 protein produced a marked decrease in FSH-induced progesterone production, but had no effect on FSH-stimulated oestradiol production, suggesting that BMP15 is a selective modulator of FSH function.

Genetic variation and mutations in the ovine BMP15 gene

The BMP15 gene (*BMP15*) of sheep maps to the X chromosome, and includes an 1179 bp coding sequence structured in two exons, and separated by a 5.4 kb intron. This produces a 393 amino acid residue pre-propeptide and a 125 amino acid mature peptide (Galloway et al., 2000). Like GDF9, BMP15 consists of three parts: a signal peptide (the pre-region), a large precursor segment with a chaperone function (the pro-region), and a mature domain at the carboxy-terminal (the mature region) (Chang et al., 2002; Liao, Moore, & Shimasaki, 2004). The molecular weight of mature BMP15 is 44,900 Da.

Both male and female mice lacking a functional *BMP15* are fertile, although sub-fertility is observed in females. While follicular growth appears normal, ovulation and the fertilisation of oocytes are impaired (Yan et al., 2001). While no apparent effect on ovulation rate or litter-size was observed in mice heterozygous for inactive copies of *GDF9* or *BMP15* alone, mice heterozygous for inactive copies of both *BMP15* and *GDF9* had smaller and less frequent litters than control mice. This effect was even more dramatic in *BMP15* knockout mice that were also heterozygous for the inactive *GDF9*. In these animals, follicular growth appeared normal, but fertilisation of released oocytes was dramatically reduced due to disruption of the cumulus cell-oocyte complex. Many oocytes were recovered with few or no cumulus cells attached. In some animals, this effect was severe enough to cause infertility. Yan et al. (2001) reported that homozygous *BMP15* knock-out female mice were sub-fertile, with reduced litter-size compared to heterozygous and wild-type females. They also believed that the *BMP15* knockout mice exhibited reduced fertility due to defects in ovulation and embryo development. It is known that the overexpression of mouse *BMP15* in oocytes does result in a higher reserve of antral follicles due to rising folliculogenesis, but concomitantly the atresia rate is increased in the transgenic mice (McMahon et al., 2008). The importance of *BMP15* in sheep fertility was confirmed with the identification of five separate point mutations in the mature BMP15 coding

region (Davis, 2005), and these were subsequently revealed to be associated with increased ovulation rate and litter-size in sheep (Hanrahan et al., 2004). There are now many other known nucleotide sequence variants of BMP15, some of which can be considered to be mutations, while others have more benign effects. Table 2 summarises the better known mutations, while a comprehensive description of nucleotide variation can be found at Ensembl (https://asia.ensembl.org/Ovis_aries/Gene/Variation_Gene/Table?db=core;g=ENSOARG00000009372;r=X:50970938-50977454;t=ENSOART00000010201). Figure 4 is a graphical representation of the currently identified nucleotide sequence variation in the gene.

Table 2 Bone morphogenetic protein 15 gene (*BMP15*) nucleotide changes/mutations and their effects on litter-size

Name	Nucleotide sequence change*	Reference number	Amino acid change	Effect on ovulation rate and litter-size	Reference
B1	ENSOART00000010201.1:c.31_33del	rs592773279	p.Leu11del	No known phenotypic effect.	(Hanrahan et al., 2004)
				The absence of this deletion increase fertility	(Guo et al., 2004)
FecX ^{Bar}	c.302_304delCTA, c.301G > T, c.310insC	-	p.Ala101CysfsTer113	Ovulation rate increases by +0.7 ova and litter- size by +0.3 lambs.	(Lassoued et al., 2017)
FecX^{G}	c.718C>T	-	p.Glu239ter-premature stop codon	The effect on ovulation rate in heterozygous ewes is +0.77 ± 0.537 in Belclare sheep and +1.18 ± 0.387 for Cambridge sheep. Homozygous ewes are sterile.	(Hanrahan et al., 2004)
FecX^{B}	c.1100T>G	-	p.Ser367Ile	The effect on ovulation rate in heterozygous was +2.38 ± 0.549 in Belclare ewes, and homozygous are sterile.	(Hanrahan et al., 2004)
FecX^{I}	c.897A>T	-	p.Val299Asp	Increase the number of lambs born per ewe by 0.6, however homozygous ewes are sterile.	(Galloway et al., 2000)
					(Davis, Dodds, McEwan, & Fennessy, 1993)
FecX^{H}	c.873C>T	-	p.Glu291ter stop codon in the place of glutamic acid	Increased ovulation rates in heterozygous ewes +1.0 and litter-size by +0.6 and sterility in homozygous Romney ewes.	(Galloway et al., 2000) (Davis, 2005)
FecX^{L}	c.963G>A	-	p.Cys321Tyr	Increased ovulation rate and sterility in heterozygous and homozygous ewes respectively	(Bodin et al., 2007)
FecX^{R}	c.487_503del	rs421419167	p.Trp163AsnfsTer55 Premature stop codon	Increased prolificacy and sterility in heterozygous and homozygous ewes respectively	(Martinez-Royo et al., 2008)
				Heterozygous ewes present 0.63 and 0.35 extra ovulations and additional lambs per lambing adult ewe respectively	(Lahoz et al., 2011)
FecX^{Gr}	c.950C>T	-	p.Thr317Ile	Increased litter-size and ovulation rate in French Grivette sheep.	(Demars et al., 2013)
FecX^{O}	c.1009A>C	-	p.Asn337His	Responsible for the highly prolific phenotype in the Olkuska breeds	
V135G	c.404T>G		p.Val135Gly	No known phenotypic effect.	
L110L	c.330C>T		p.110Leu–No change	No known phenotypic effect.	
A77A	c.231T>G		p.110Ala–No change	No known phenotypic effect.	
P101A	c.301G>C		p.Pro101Ala	No known phenotypic effect.	

[+]Nucleotide positions relative to GenBank NC_019484

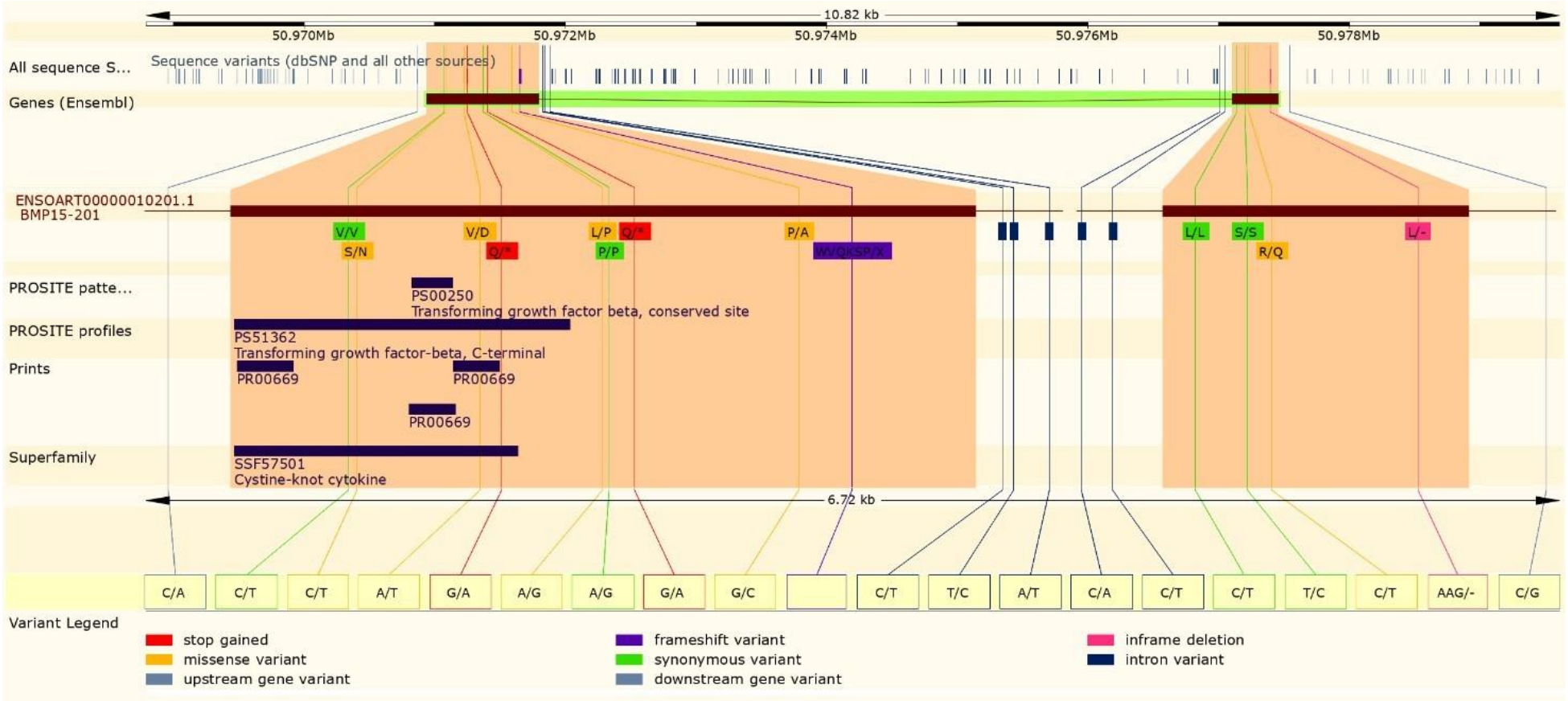


Figure 4 Ensembl image file of ovine *BMP15* sequence variation (https://asia.ensembl.org/Ovis_aries/Transcript/Variation_Transcript/Image?db=core;g=ENSOARG00000009372;r=X:50970938-50977454;t=ENSOART00000010201)

1.5.3 Bone Morphogenetic Protein Receptor Type 1B (BMPR1B) gene (*BMPR1B*, Ensembl: ENSOART00000018678.1, also known as *Alk6, SKR6, ALK-6, AMDD*, *BDA2, BDA1D, CDw293, Acvrlk6, BMPR-1B, BMPR-IB, CFK-43a, AI385617* and *AV355320*)

In the early 1980s, research conducted on fertility and litter-size in Merino sheep revealed that there was a major autosomal gene with a positive effect on ovulation rate and on prolificacy. Those sheep that received one copy of the so-called, but unidentified 'Booroola gene' (called B or FecB) from each of their parents produced 1.5 more ovules and one lamb more than any other sheep in each lambing (Davis et al., 1982). In 1993, the first DNA marker test for the Booroola gene revealed that this gene was located on chromosome 6. The test was performed using three gene markers that were located close to each other on the chromosome and that provided 90% accuracy in predicting the phenotype (Montgomery et al., 2001). The ovarian phenotype in homozygous ewes (BB) is completely different from ewes homozygous for *BMP15* or *GDF9*. The most important characteristic of homozygous ewes (BB) is the larger size and number of ovarian follicles than other genotypes. Mature and ovulated follicles in homozygous (BB) and heterozygous (B+) sheep have a smaller diameter than in the wild-type homozygous (++) sheep. Smaller ovarian follicles in BB ewes have fewer granulosa cells than ++ ewes (McNatty et al., 2005). Thus, the number of granulosa cells from all ovarian follicles and the total amount of steroid or inhibin output from the ovary of the homozygous (BB) or heterozygous (B+) ewes are similar to the wild-type homozygous (++) (Wilson et al., 2001). The most important feature of ewes carrying the Booroola gene is the small size of ovum comparing to those ewes without this gene. The non-carrier homozygous ewes of this gene have an average of one to two ovum with a diameter of seven millimetres, heterozygous ewes for this gene have three to four ova (four to five millimetre in diameter), and homozygous ewes with Booroola gene in each cycle of more than five ova (three to five millimetres in diameter) (Davis et al., 1982). The reduction in cell proliferation activity and increase the in expression of the main markers responsible for the follicular maturity during the follicle growth in the ovary, is characterized by the development of aromatase activity and LH receptors by the granulosa cells of the antral follicles at markedly smaller diameters than in wild-type ewes. The most important effect of the Booroola gene is in increasing FSH levels, which are much higher in homozygous ewes than wild-type ewes (Elsen et al., 1991). The increase in FSH is due to an increase in hormone secretion from the pituitary gland and ovarian follicles (Lundy et al., 1999). Young et al. (2008) investigated whether the Booroola gene directly or indirectly led to an increase in FSH levels. They found that the pituitary cells of the ewes carrying the Booroola gene had a higher sensitivity to the BMP hormone group than the wild-type, and these hormones led to a significant reduction in the secretion of FSH. The similarity in the size of the pituitary gland, the number of cells in the gland, the number of cells containing FSH and LH in the ewes carrying Booroola and wild-type ewes and the high sensitivity of the pituitary cells of the ewe carrying the

gene to the BMP group indicates that Booroola gene does not directly increase FSH but acts through the effects of BMP or GnRH hormones (Young et al., 2008).

Over time the gene underpinning the Booroola phenotype was identified and it has many names in the literature. The protein is now called the Bone Morphogenetic Protein Receptor Type 1B and the gene is *BMPIRB*.

Genetic variation and mutations in the ovine BMPR1B gene

Many sequence variants of *BMPR1B* have been described, some of which can be considered to be mutations, while others have more benign effects (Table 3).

It was found that BMPR1B-deficient females are infertile due to a constellation of defects, including irregular oestrus cyclicity, impaired pseudo-pregnancy responses, severe defects in cumulus cell expansion, and insufficient uterine endometrial gland development (Yi et al., 2001). BMPR1B knock-out leads to infertility in mice due to a block in folliculogenesis at the primary stage and increased fertility in sheep (Baur et al., 2000; Yi et al., 2001). Various studies (Mulsant et al., 2001; Davis et al., 2006; Polley et al., 2010) have been published on the importance of *BMPR1B* mutation in sheep prolificacy and they have proposed that no variation was observed in the expression levels of the mutated gene. Hence, the regulation of follicular development appears to be due to changes in the signal transduction pathway (Yi et al., 2001). BMPR1B serves as a potent receptor for various BMP factors including BMP15 (Ten Dijke et al, 2003). *BMP15, GDF9,* and *BMPR1B* modulate the effect of FSH on antral follicles.

A comprehensive description of nucleotide variation can be found at Ensembl (https://asia.ensembl.org/Ovis_aries/Gene/Variation_Gene/Table?db=core;g=ENSOARG00000017161;r=6:29361947-29448079;t=ENSOART00000018678). Figure 5 is a graphical representation of the currently identified nucleotide sequence variation in the gene.

Table 3 BMPR1B (Bone Morphogenetic Protein Receptor Type 1B) nucleotide changes/mutations and their effects on litter-size

Name	Nucleotide sequence change*	Reference number	Amino acid change	Effect on ovulation rate and litter-size	Reference
FecB, Fecundity Booroola,	ENSOART00000018678.1:c.746A>G		p.Arg249Glu	Increase ovulation rate and litter-size in most sheep breeds around the world (Hyper-prolific).	(Piper & Bindon, 1983) (Piper, Bindon, & Davis, 1985) (Fabre et al., 2003) (Souza et al., 2001) (Chu et al., 2007) (Chu et al., 2011) (Davis et al., 2006) (Davis et al., 1982) (Wilson et al., 2001) (Mulsant et al., 2001) (Polley et al., 2010) (Kumar et al., 2008) (Roy et al., 2011) (Zuo et al., 2013) (Mahdavi et al., 2014) (Jia et al., 2005) (Yan et al., 2005) (Liu et al., 2003) (Tian et al., 2009) (Zuo et al., 2013) (Fogarty, 2009)
M64I	ENSOART00000018678.1:c.360G>A	rs428753381	p.Met64Ile	Unknown	(Heaton et al., 2017)
T345N	ENSOART00000018678 c.1180A>C		p.Thr345Asp	Unknown	(Heaton et al., 2017)
	g.66496G>A		p.Thr306 – No change	Unknown	(Abdoli, et al., 2018)

[+]Nucleotide positions relative to GenBank NC_019463.1

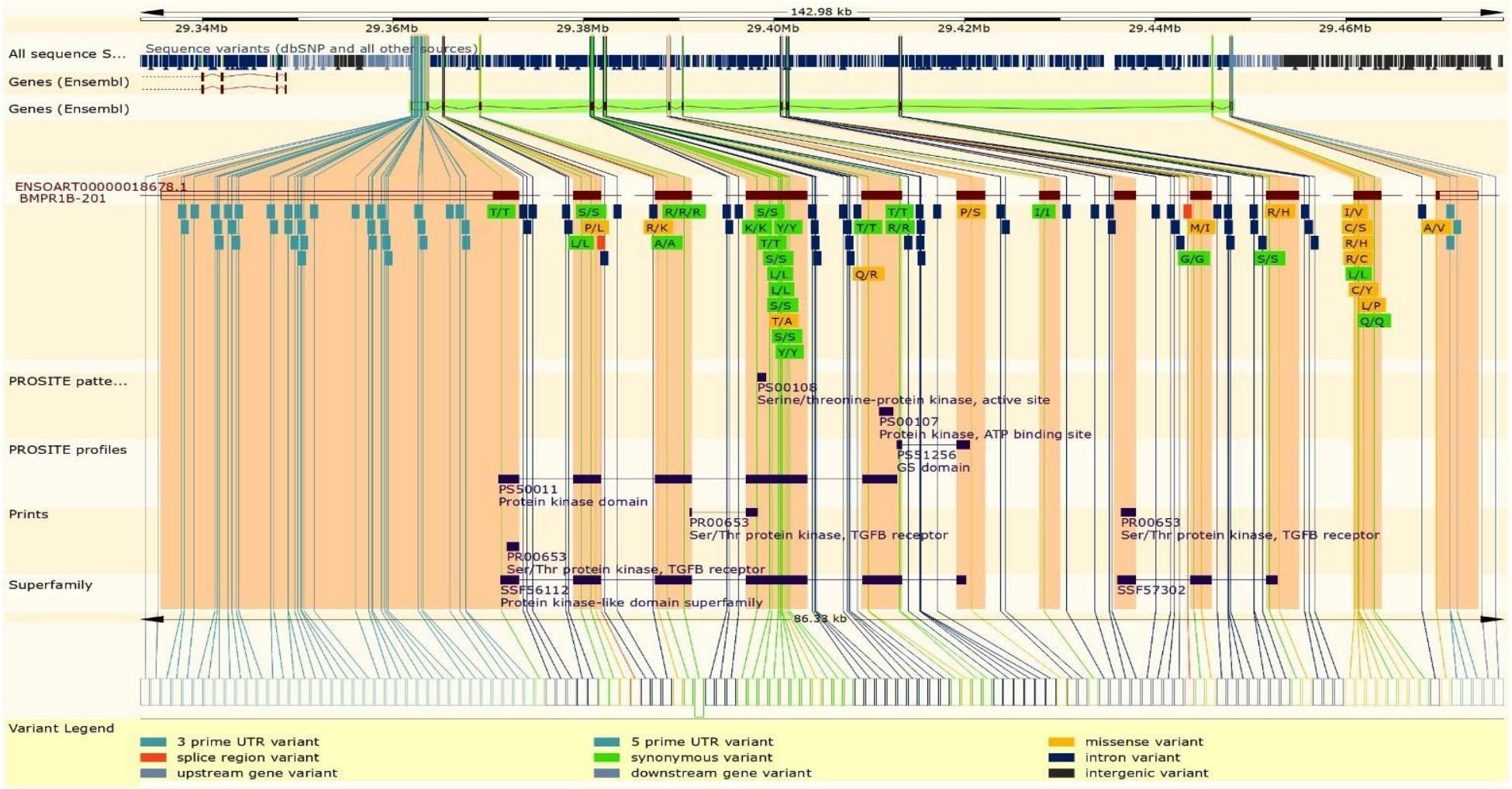


Figure 5 Ensembl image file of ovine *BMPR1B* sequence variation

https://asia.ensembl.org/Ovis_aries/Transcript/Variation_Transcript/Image?db=core;g=ENSOARG00000017161;r=6:117031472117031472;t=ENSOART00000018678;v=rs424181501;vdb=variation;vf=18621617

1.6 Sheep studied and statistical models used in this thesis

The sheep breeds investigated in this study were Finnish Landrace, Finnish Landrace × Texel-cross, NZ Romney, Coopworth, Perendale, White Dorper, Dohne, Merino, Wiltshire, Texel, Corriedale, and Polwarth. There were also other 'composite sheep' of undefined breed background, but based on NZ Romney-type genetics. These breeds were chosen based on their variability in prolificacy, but for all of them understanding what controls fertility would be of interest to the New Zealand sheep industry. The amount of available data about fertility varied from breed to breed, but included data for the average number of lambs born per ewe, the ewe's age at lambing and pedigree, up to a maximum depth of five consecutive generations. The investigation of the variation detected in the genes studied and their association with litter-size was carried out in only three sheep groups including NZ Finnish Landrace, Finnish Landrace × Texel-cross and composite sheep. For the association study, best linear unbiased prediction (BLUP) has proven itself to be an efficient method for genetic evaluation of domestic livestock. The ASREMEL software used in this thesis was used to estimate the additive and dominance effects of SNPs using two models: animal models and sire models. In the sire models, the sires were evaluated using progeny records and it was is assumed that all mates are of similar genetic merit and this can result in bias in the predicted breeding values if there is preferential mating, but in animal models, full pedigree is considered in the model and it includes all animals including those with records and without records. The main advantage of the sire models is that the number of equations solved is less than in the animal models, since only sires are evaluated.

Blood samples from these breeds were made available by the Gene-Marker Laboratory at Lincoln University. The Table 4 summarises the wide variety of sheep breeds in New Zealand. The main breeds are: 1) the New Zealand Romney (NZ Romney), with a lambing percentage (lambs weaned to ewes mated) of 90 - 140 (one of the most popular breeds in NZ and constitutes more than 60% of the National flock), 2) the Perendale with medium fertility with a lambing percentage of more than 115 and that constitutes 10 to 15% of National flock, 3) the Coopworth makes up the second largest flock in New Zealand (13%) which is known as prolific sheep with a lambing percentage of 110 - 160 and that is a stabilised cross between the Romney and the Border Leicester, and 4) the Merino sheep with an average lambing percentage of 90% and constitutes only about 6% of the national flock . There are also Texel sheep, Finnish Landrace (Finn) sheep (one of the most fertile breeds in New Zealand with a lambing percentage around 260), the Wiltshire (another prolific breed with a lambing percentage over 180%), the Corriedale with an average lambing percentage of 90 - 130%, and the Polwarth with an average lambing percentage of 100 -120%

Table 4 Description of different sheep breeds in New Zealand (http://www.therural.co.nz/livestock/sheep- breeds-in-new-zealand, 2017)

Breed	Purpose	Features
Borderdale	Dual-purpose	Lean meat, strong long & medium-fine wool. Good milk production. Easy lambing. Mostly in
Border	Dual-purpose	Increases other breed's fertility through crossbreeding. Strong wool, good fleece weight, lean
Cheviot	Dual-purpose	Small, short-legged, polled. Good foragers, fine-grained meat and strong, bulky, low lustre wool. Used for crossbreeding as-as terminal sires.
Coopworth	Dual-purpose	Medium-large, high production. Lean, tender meat. Strong, course, long wool.
Corriedale	Dual-purpose	Medium sized, good longevity, quality meat, and medium-fine wool. Fertile often crossed with Romney or Perendales.
Dorper	Meat	Two variations - Black-headed Dorper and White Dorper. High growth rates, self-shedding. Produces lambs three times in two years.
Dorset Down	Meat	Medium-large, high growth rate. Lean, high yielding carcass.
Dorset Horn	Meat	Curled horns. Hardy, large breed. High growth rate, produce early and out-of-season lambs.
Drysdale	Multi-purpose	Horned. Medium-large, high quality, long, strong, hairy wool that is used for carpet
East Friesian	Multi-purpose	High milk production, high lamb growth rates & lean carcass. White, strong, bulky wool.
English	Multi-purpose	Large, hardy, fertile. Pale meat. Long, lustrous, heavy wool
Finn sheep	Multi-purpose	Fertile, fine wool, lean carcass. Used for crossbreeding.
Half-bred	Multi-purpose	Medium sized, with fine-medium wool. Medium-grained, tender meat. A cross of Merino and long-wool sheep.
Hampshire	Meat	Lean, sweet, high-quality meat. Large, rapid growth rate. Downy wool.
Lincoln	Wool	Strong, long, course wool. High micron and tensile wool. Hardy, suitable for wet and cold
Merino	Wool	Hardy, medium sized. Fine, high-quality wool. Mostly in the South Island.
NZ Romney	Multi-purpose	Fertile, easy lambing. Medium-large. Large, lean lambs. Heavy, medium lustre wool.
Oxford	Meat	Large, high growth rate, early maturing. Large, lean carcass.
Perendale	Multi-purpose	Medium sized, hardy, easy care. Suitable for the hill country. High growth rate. Long, low
Poll Dorset	Meat	Breed twice a year. White, dense, fine wool. Low-fat meat.
Polwarth	Multi-purpose	Breed year-round Medium sized. Fine-grained meat. High yielding, fine wool. Mainly in the
Ryeland	Multi-purpose	Ideal for small farmers. Medium sized, docile. Medium, dense wool.
Shropshire	Meat	Hardy, medium sized. Sires for terminal crossing.
Southdown	Meat	Medium sized. High growth rate, fine-grained, sweet, red meat.
South Dorset	Meat	Early maturing, do well in drought conditions. Prime lamb meat.
South Suffolk	Meat	Large, high growth rates. High yielding carcass.
Suffolk	Meat	High growth rate, large, hardy. Lean carcass.
Texel	Meat	Hardy, used in composite breeding. Course-grained meat. Lean carcass.

1.7 Aims of this thesis

While the relationship between variation in *GDF9, BMP15* and *BMPR1B* and variation in fertility is quite well understood in some breeds, little is known about the genes in other breeds, especially those commonly farmed in New Zealand. Accordingly, this study focused on a variety of breeds, and breeds that span a spectrum of fertility. Fertility is a key determinant of profitability in NZ farming systems, and variation in these genes is already being used in some breeds to improve reproductive performance.

If variation exists in *GDF9, BMP15* and *BMPR1B* in the common New Zealand breeds, then that variation may be useful for improving fertility. This will be undertaken using a combination of polymerase chain reaction - single-strand conformation polymorphism (PCR-SSCP) analyses and DNA sequencing to detect the sequence variation, and then a variety of statistical analyses to ascertain if the variation can predict variation in fertility.

Chapter 2

Genetic variation in the growth differentiation factor 9 gene (*GDF9*) in New Zealand sheep

2.1 Introduction

New Zealand (NZ) is a major exporter of sheep meat, predominantly lambs, or sheep that are under one year of age. In the 2017 season, the NZ farmer organization Beef and Lamb NZ (B+LNZ), reported that 23.7 million lambs had been tailed (lamb tails are docked to mitigate flystrike), with a national lambing percentage of 127.2% (i.e. an average of 1.272 lambs per ewe mated). The number of these lambs that survive to weaning determines the amount of meat produced per ewe, and thus, NZ's export meat production is to a great extent determined by lambing performance. This explains the ongoing research emphasis on improving fertility, fecundity, and lamb survival.

Two important traits with high economic value to sheep production are, therefore, ewe ovulation rate and litter-size (Notter, 2008). Ovulation rates differ in different breeds, and the range is from one egg per ovulation (as is typical for the Texel or Suffolk breeds), up to ten eggs per ovulation for prolific breeds such as the Booroola Merino, or Finnish Landrace sheep (Souza et al., 2001). Factors affecting ovulation rates in individual ewes include their genetics, stress levels, weight, and age (Kareta te al., 2006). With respect to genetics, the Finnish Landrace breed has been used as a source of genetic material around the world to cross into other sheep breeds to increase fecundity. Understanding the factors that affect ovulation rates is not only important from an animal production perspective, but also enables improved understanding of animal infertility and other genetic disorders that affect reproductive performance (Jansson, 2014).

Genetic improvement in ovulation rate in sheep is slow because it is only expressed in one gender (sex limited trait), and because an accurate record of the trait, for any given ewe, can only fully be achieved at the end of her reproductive life. Attention has, therefore, focussed on the genes that might underpin variation in fertility, this in the hope that when identified, these genes will enable sheep with superior reproductive performance to be selected for breeding. In this context, there have been many studies in sheep describing how members of the transforming growth factor ß (TGF ß) superfamily and their related cell-surface receptors are essential intra-ovarian regulators of development and/or of ovulation rate (Galloway et al., 2000; Mulsant et al., 2001). The TGF ß superfamily includes more than 35 members, a number of which appear to be critical for regulating fertility (Juengel et al., 2004). A TGF ß superfamily member that has received considerable attention is growth differentiation factor 9 (GDF9), or FecG (McNatty et al., 2005). The GDF9 gene (*GDF9*) is

expressed from the primary stage of follicular development (McGrath et al., 1995). It is an autosomal gene located on ovine chromosome 5.

Various sequence variations have been described in the ovine *GDF9*. For example, 'Vacaria' ($FecG^{V}$), or c.943C>T/p.Arg315Cys (Souza et al., 2014) and c.1111G>A/p.Val371Met (Våge et al., 2013), are variations in *GDF9* that appear to have an additive effect in increasing litter-size. Contrastingly, 'High Fertility' ($FecG^{H}$), or c.1184C>T/ p.Ser1184Phe (Hanrahan et al., 2004) and 'Thoka' ($FecG^{T}$), or c.750G>A/p.Ser427Arg (Nicol et al., 2009), could be considered to be mutations, as they increase prolificacy in the heterozygous state, but are associated with sterility in homozygous ewes. Another nucleotide substitution, c.994G>A/p.Val332Ile (Hanrahan et al., 2004), has not been reported to have any association with fecundity, but more analysis will be needed to confirm this result.

Increased knowledge about the genes that affect fertility and litter-size in sheep has the potential to increase profitability in sheep production systems. Accordingly, a better understanding is required of *GDF9* variation in NZ's most common maternal sheep breeds (e.g., the NZ Romney, Perendale, Coopworth, and out-crosses of those breeds). These breeds may have potentially more benign variation in *GDF9*, but a variation that if selected for, would allow us to better control and increase the number of lambs born per ewe, per year, on individual farms. This might enable a better 'matching' of lambing performance to feed supply, and potentially the ability to finish lambs on the farm to a weight where they can be slaughtered for export, thereby improving the resilience of the system. To develop the tools to undertake this research into common NZ maternal breeds, composite sheep that have one of the common NZ maternal breeds in their lineage, and which have been described as having higher fertility, were investigated.

2.2 Materials and method

All research involving animals was conducted under authority from the Animal Welfare Act 1999 (NZ Government). And the collection of sheep blood drops by the nicking of their ears was covered by Section 7.5 Animal Identification, in: *Code of welfare: sheep and beef cattle (2016); a code of welfare issued under that act.* This process is considered to be a regular practice in farm management system, and cause little or no harm to animal, therefore no formal ethics review needed in this study.

Blood samples and DNA purification

One thousand and sixty-four sheep were studied. The blood samples were obtained from different farms. The Finnish Landrace breed (n = 164), and Finnish Landrace × Texel-cross sheep (n = 118) and one of the composite sheep (n = 189) belonged to one farm (farm 1) located in the North of Canterbury. Composite sheep are sheep bred from a wide variety of genetic backgrounds based on selection for key production traits. Their background is typically very diverse, and in the case of the

sheep described here will include at very least NZ Romney, Texel, East Friesian and Finnish Landrace sheep based on what is known about the history of the flock. The breed proportion is unknown and likely variable from sheep to sheep. These sheep were primarily bred for lamb/meat production and not wool or milk, using the NZ eBV-based system known as Sheep Improvement Limited (SIL).

There were another 266 composite sheep of undefined breed background, but based on NZ Romney type genetics, and derived from another three farms (n = 220, 38, and 76, from farms 2-4 respectively). Besides these, blood samples were randomly collected from different farms around New Zealand in South Island for NZ Romney sheep (n = 48), Coopworth (n =24) and Perendale (n = 24), White Dorper (n = 24), Dohne (n = 24), Merino (n = 24), Wiltshire (n = 24), Texel (n = 24), Corriedale (n = 24) and Polwarth (n = 24). All the composite sheep were identified as potentially containing some Finnish Landrace genetics. The percentage of Finnish Landrace in the composite sheep from two farms (farm 1 and farm 2) ranged 12.5% up to 50 %, but it was not known precisely how much Finnish Landrace genetics was in these sheep.

Blood from the sheep investigated was collected onto FTA cards from a small incision in the ear of the sheep. DNA was extracted from the blood samples by punching a 1.2 mm disc from the FTA card, followed by genomic DNA purification using a two-step procedure described by Zhou et al. (2006). To begin this process, the FTA card punch was placed in tubes containing 200 µL of 20 mM NaOH and left for 20 to 30 minutes at 62 °C, or until the disk became white. All the liquid was then removed by aspiration and the disk equilibrated in 200 µL of 1× TE-1 buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). After this, the liquid was again removed, and the disks were left overnight to air dry in the tubes.

PCR amplification and PCR-SSCP analysis of GDF9

A Polymerase Chain Reaction - Single Strand Conformation Polymorphism (PCR-SSCP) approach was used to search for sequence variation in a 395-bp amplicon of the GDF9 gene. The PCR primers used were 5'-ATAAGCGATTGAGCCATCAGG-3' (forward primer) and 5'-GCTGAGGGTGTAAGATCGTC-3' (reverse primer). The primers were designed based on GenBank sequence AF07854.2 to amplify a fragment that spanned nucleotides 3826 to 4221 of the AF07854.2 sequence of the exon 2 region and encompassed nucleotide variation reported previously in the literature that had an association with litter-size. These SNPs include c.943C>T (Souza et al., 2014), c.1111G>A (Mullen et al., 2014; Våge et al., 2013), c.1184C>T (Galloway et al., 2000; Hanrahan et al., 2004), and c.1279A>C Nicole et al., 2009). The PCR amplifications were performed in a 15-µL reaction containing the genomic DNA on one 1.2-mm punch of FTA card, 0.25 µM of each primer, 150 µM of each dNTP (Bioline, London, UK), 0.3 mM Mg2+, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1× reaction buffer supplied with the enzyme. Amplification was undertaken as follows: initial denaturation at 94 °C for

2 min, followed by 35 cycles 94 °C for 30 s (denaturation), 59 °C for 30 s (annealing), and 72 °C for 30s (elongation), with a final extension step at 72 °C for 5 min.

The amplicons obtained from the PCR reactions were visualized by electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels, using 1x TBE buffer (89 mM Tris, 89 mM boric acid,2mMNa2EDTA), containing 200 ng/mL ethidium bromide. A 2µLaliquot of PCR product was added to 2µL of loading dye (0.2% bromophenol blue, 0.2% xylene cyanol, 40% (w/v) sucrose) and the gels were run at a constant 10 V/cm for 10 min., prior to visualization by UV trans-illumination at 254 nm.

For SSCP analysis, a 0.7 µL aliquot of each amplicon was mixed with 7 µL of loading dye (98% Formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol), and after denaturation at 95 oC for 5 minutes, the samples were rapidly cooled on wet ice and immediately loaded on to 16 cm × 18 cm, 12% acrylamide: bisacrylamide (37.5:1; Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad), at 350 V for 18 hours at 7 oC in 0.5x TBE buffer. The DNA fragments were visualized using a silver nitrate staining method (Byun et al. 2009). Briefly, the gels were bathed in a solution of 10% ethanol, 0.5% acetic acid and 0.2% AgNO3 for 10 minutes. Next, the gels were rinsed with distilled water then developed with a solution of 3% NaOH and 0.1% HCOH until dark-staining bands appeared on the yellow background of the gel.

Sequencing of variants and sequence analyses

PCR amplicons representing different banding patterns from sheep that appeared to be homozygous were sequenced in triplicate in both directions at the Lincoln University DNA sequencing facility, to confirm that the variants detected represented unique DNA sequences. Variants that were only found in heterozygous sheep were sequenced using an approach described by (Gong et al., 2011). Briefly, a band corresponding to each variant was excised as a gel slice from the polyacrylamide gel, macerated, and then used as a template for re-amplification with the original primers. This second amplicon was then sequenced directly. Sequence alignments, translations, and comparison were carried out using Geneious version 5.5.3, (http://www.geneious.com, Biomatters, New Zealand) (Kearse et al., 2012). The resulting sequences were displayed using the ChromasPro software (Technelysium, 1996).

Statistical analyses

Allelic and genotypic frequencies were calculated in R programming software (Team, 2013) for the Finnish Landrace, the Finnish Landrace × Texel-cross, NZ Romney, White Dorper, Dohne, Merino, Perendale, Coopworth, Wiltshire, Texel, Corriedale, Polwarth, and four different composite sheep flocks from four separate farms.

The calculation of variant and genotype frequencies, and Hardy-Weinberg equilibrium were performed using the methods test with likelihood-ratio as the test statistic, as appropriate for a sample containing multiple alleles as described by (Engels, 2009).

2.3 Results

PCR-SSCP analysis of the 395 bp amplicon of *GDF9* exon 2 in the different sheep breeds, revealed three banding patterns (named *A*, *B,* and *C*), and six genotypes of these banding patterns (*AA*, *AB, AC, BB, BC* and *CC*) (Figure 6). Sequencing confirmed that the three variants were unique DNA sequences.

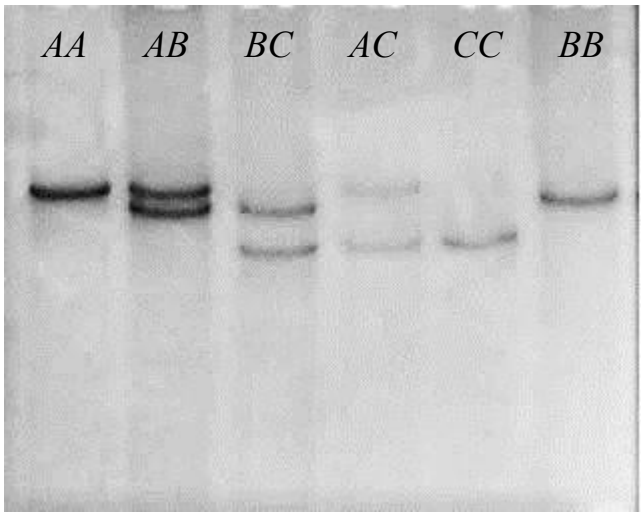


Figure 6 Six different Polymerase Chain Reaction – Single Strand Conformation Polymorphism (PCR-SSCP) patterns (AA, AB, BC, AC, CC and BB) for an exon 2 fragment of GDF9 in New Zealand (NZ) sheep breeds (Finnish Landrace, Finnish Landrace x Texel-cross, Romney, and composite sheep).

Sequence analyses of the three variants revealed three nucleotide variations: c.978A>G, c.994G>A and c.1111G>A. The nucleotide substitution C.978A>G was a silent substitution (i.e., would result in no amino acid change). The relationship between the occurrence of these nucleotide variations and the three variants is detailed in Figure 7. The nucleotide substitution c.994G>A has been reported previously (Hanrahan et al., 2004) and would result in a substitution of valine with isoleucine (p.Val332Ile). The c.1111G>A nucleotide substitution has also been reported previously ((Hanrahan et

al., 2004; Våge et al., 2013), and upon translation would result in p.Val371Met (Figure 7). The three nucleotide substitutions detected in this study have been described in earlier studies, and no new variation was found in NZ sheep breeds.

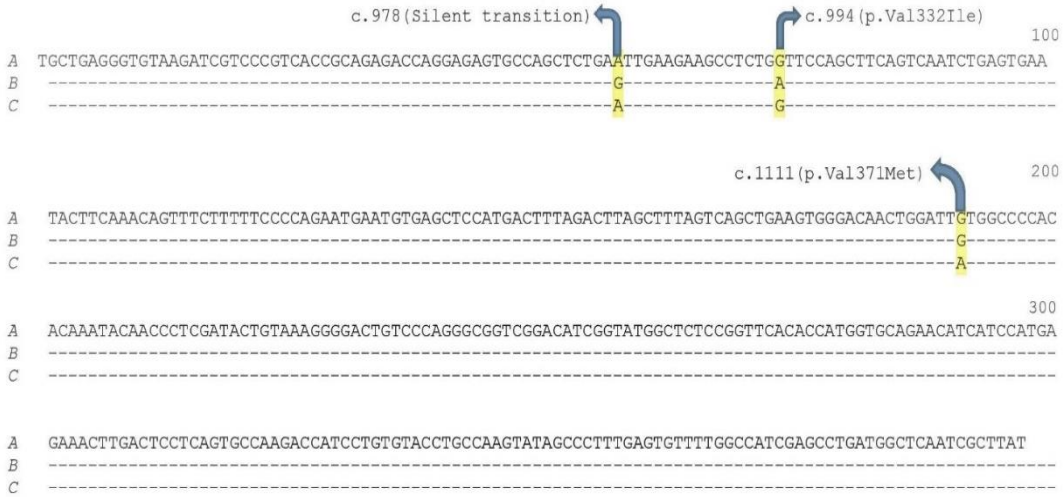
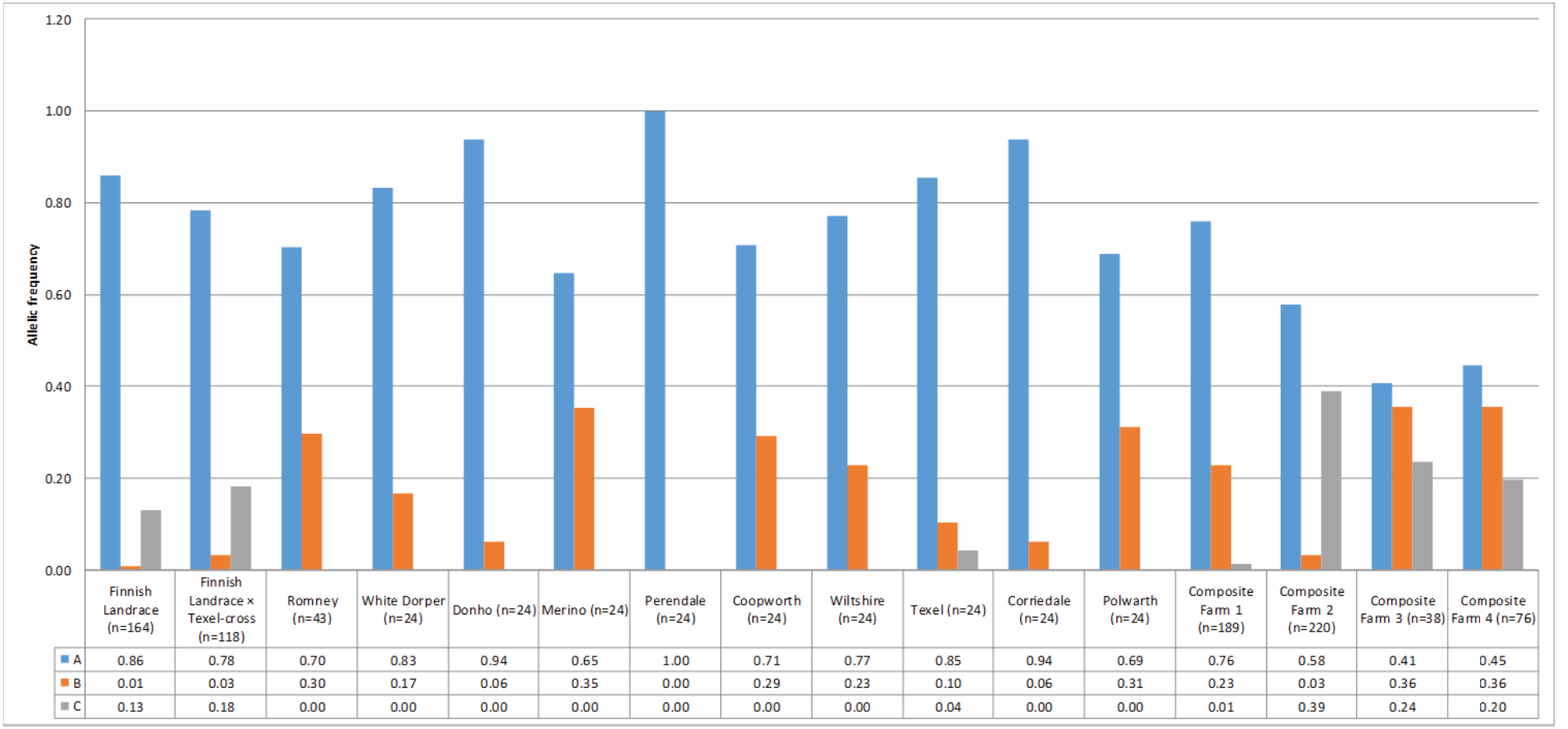


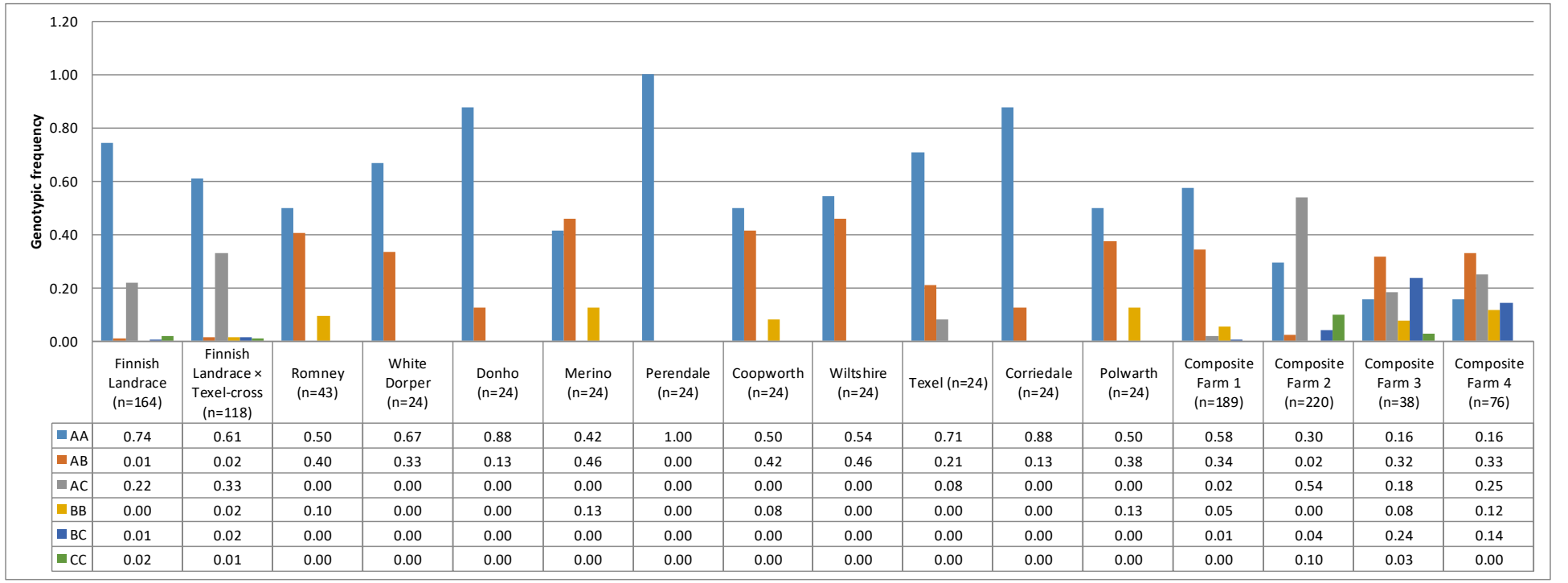
Figure 7 Nucleotide sequences of Growth Differentiation Factor 9 gene (*GDF9*) variants A - C.

Nucleotides in the coding region are shown in uppercase, while those outside the coding region are in lowercase. The position of the nucleotide variation marked above the sequences, and those that would result in amino acid changes are indicated.



	Finnish Landrace (n=164)	Finnish Landrace × Texel-cross (n=118)	Romney (n=43)	White Dorper (n=24)	Donho (n=24)	Merino (n=24)	Perendale (n=24)	Coopworth (n=24)	Wiltshire (n=24)	Texel (n=24)	Corriedale (n=24)	Polwarth (n=24)	Composite Farm 1 (n=189)	Composite Farm 2 (n=220)	Composite Farm 3 (n=38)	Composite Farm 4 (n=76)
A	0.86	0.78	0.70	0.83	0.94	0.65	1.00	0.71	0.77	0.85	0.94	0.69	0.76	0.58	0.41	0.45
B	0.01	0.03	0.30	0.17	0.06	0.35	0.00	0.29	0.23	0.10	0.06	0.31	0.23	0.03	0.36	0.36
C	0.13	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.01	0.39	0.24	0.20

(A)



	Finnish Landrace (n=164)	Finnish Landrace × Texel-cross (n=118)	Romney (n=43)	White Dorper (n=24)	Donho (n=24)	Merino (n=24)	Perendale (n=24)	Coopworth (n=24)	Wiltshire (n=24)	Texel (n=24)	Corriedale (n=24)	Polwarth (n=24)	Composite Farm 1 (n=189)	Composite Farm 2 (n=220)	Composite Farm 3 (n=38)	Composite Farm 4 (n=76)
AA	0.74	0.61	0.50	0.67	0.88	0.42	1.00	0.50	0.54	0.71	0.88	0.50	0.58	0.30	0.16	0.16
AB	0.01	0.02	0.40	0.33	0.13	0.46	0.00	0.42	0.46	0.21	0.13	0.38	0.34	0.02	0.32	0.33
AC	0.22	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.02	0.54	0.18	0.25
BB	0.00	0.02	0.10	0.00	0.00	0.13	0.00	0.08	0.00	0.00	0.00	0.13	0.05	0.00	0.08	0.12
BC	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.04	0.24	0.14
CC	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.03	0.00

(B)

Figure 8 (A) The observed variant and (B) genotype frequencies for the variants of an exon 2 fragment of *GDF9* in New Zealand (NZ) Finnish Landrace, Finnish Landrace x Texel-cross, and composite sheep.

The frequencies of the variants and genotypes are summarised in Figure 8. Nearly half of the sheep studied were of genotype *AA,* and the frequency of *CC* in the Finnish Landrace and Finnish Landrace × Texel-cross sheep was 2% and 1% respectively. This investigation revealed that all of the substitutions: variant A, c.978A>G, c.994G>A were found in most of the breeds studied on the different farms. None of the composite sheep from farms 1 and 4, NZ Romney, White Dorper, Dohne, Merino, Perendale, Coopworth, Wiltshire, Texel, Corriedale, Polwarth were *CC*. No *BB* was found in the Finnish Landrace, White Dorper, Dohne, Perendale, Wiltshire, Texel, Corriedale, and composite sheep from Farm 2. The frequency of *AB* was very low in the Finnish Landrace and Finnish Landrace × Texel-cross sheep (1% and 2% respectively), while the highest frequency of *AB* was 46%, in the Merino and Wiltshire. It is apparent from Figure 8, that *AC* is present only in the composite sheep, Finnish Landrace, Texel and Finnish Landrace × Texel-cross sheep. All the genotypes were observed in all the composite sheep, with the exception that *BB* was not recorded on farm 2, and *CC* was not recorded on farms 1 and 4. Out of the 1064 samples, the homozygous genotype *AA* occurred most frequently (714 Samples) in all breeds, while the other two homozygous genotypes *BB* and *CC*, appeared in just 36 and 27 samples, respectively. Results of the HWE test showed only a significant deviation from equilibrium within Finnish Landrace × Texel-cross breed ($P < 0.01$).

Overall, the *A* variant was most common in the Perendale, Dohne and Corriedale sheep (100%), whereas variant *B* was most prevalent in the composite sheep from Farms 3 and 4 (36%), and variant *C* in the Farm 2 composites (39%). Only 1% and 3% of the Finnish Landrace and Finnish Landrace x Texel-cross respectively, were carriers of c.978A>G and c.994G>A. The substitution c.1111G>A (variant *C*) was detected at a very low frequency in the composite sheep from farm 1(1%). Interestingly, this nucleotide sequence variation was not detected in most breeds, including Perendale, Coopworth, NZ Romney, White Dorper, Dohne, Merino, Corriedale and the Polwarth breeds. However, it was found in the Texel sheep, but no homozygous c.1111A Texel sheep were observed.

Of the 1064 sheep genotyped, no homozygous individuals were identified for the c.994A variation in the Finnish Landrace sheep, White Dorper, Dohne, Perendale, Wiltshire, Texel, Corriedale or the composite sheep on farm 2.

All three variants of *GDF9* were found in the Finnish Landrace, Finnish Landrace × Texel-cross sheep, Texel and the composite sheep. The frequency of sheep with genotype *AA* were nearly similar in the Finnish Landrace x Texel- cross sheep and the composite sheep from farm 1 (61% and 58% respectively), while the genotype was more common in the pure Finnish Landrace sheep (74%).

2.4 Discussion

The advantage of using DNA information in breeding is that it enables us to improve the rate of genetic gain when compared with breeding programmes that only use phenotypic information (Meuwissen et al., 2001). When genes that contribute to useful traits have been characterised, DNA marker-assisted selection (MAS) can be used in breeding for improvement in traits that are either expressed later in life, or sex-limited, and/or have low heritability, such as litter-size (Dekkers, 2004).

In sheep, screening ewes for genetic variation that is known to affect prolificacy is an effective way to manage fertility in flocks. For example, studies suggest that growth differentiation factor 9 (*GDF9*), bone morphogenetic protein 15 (*BMP15*; also known as *GDF9B*) and bone morphogenetic receptor type 1B (*BMPR 1B*) are important intra-ovarian regulators of ovulation rate and thus litter-size in sheep (Galloway et al., 2000; Mulsant et al., 2001; Souza et al., 2001). Of these three factors, *GDF9* appears to have a critical role in regulating mammalian fertility, and the objective of this study was, therefore, to investigate *GDF9* sequence variation in different flocks of sheep.

In the sheep investigated, the *GDF9* was found to be variable. Three sequence variations (c.994G>A, c.978A>G and c.1111G>A) were detected in the gene for sheep from the four farms, including Finnish Landrace and Finnish Landrace x Texel-cross sheep, and other composite sheep. Six different variant genotypes (*AA*, *AB*, *AC*, *BB*, *BC,* and *CC*) were found, but the observed genotype frequencies deviated from the expected genotype frequencies (calculated based on the variant frequencies) in the Finnish Landrace × Texel-cross sheep, which could be a result of non-random mating between the sheep from two different breeds.

Variant *C* in this study has the sequence c.1111A and encodes the amino acid methionine at position 371 of GDF9. This substitution has been described previously in Cambridge and Belclare sheep (Hanrahan et al., 2004), Norwegian white sheep (Våge et al., 2013) and Finnish Landrace sheep (Mullen & Hanrahan, 2014). This substitution is associated with litter-size in the Norwegian White sheep (Våge et al., 2013), but no association with fertility was observed in the Cambridge and Belclare sheep, where it results in homozygous mutant sheep to show complete primary ovarian failure, leading to total infertility (Hanrahan et al., 2004). The presence of the c.1111A variant in the Cambridge breed is unsurprising, given the genetic contribution of the Finnish Landrace breed to the ancestors of the Cambridge breed (Mullen & Hanrahan, 2014). The c.1111A variant identified in this study was present in Finnish Landrace, Finnish Landrace × Texel-cross, Texel, and all the composite sheep. All of the composite sheep were believed to have some Finnish Landrace genetics in them, and it might therefore be appropriate to assume that the presence of the c.1111A in the composite sheep was originally from Finnish Landrace breed. That cannot however be proven. Although the c.1111A variant was detected in the Texel and composite sheep in farm 1, the frequency was very low.

Variant and genotypic frequencies for *GDF9* in various sheep breeds have been reported previously (Hanrahan et al., 2004; Mullen & Hanrahan, 2014; Våge et al., 2013). Mullen and Hanrahan (2014) reported that the frequency of c.1111A was 30% in the Finnish Landrace breed, which is higher than the variant frequency found in this study (13%) for that breed in NZ. The frequency for this sequence variant (c.1111A) in Norwegian white sheep (25%: (Våge et al., 2013)), was also more than that seen in Finnish Landrace and the Finnish Landrace × Texel-cross sheep (18%), in the current study. Heaton et al. (2017) reported that the frequency of the *GDF9* c.1111A variant was 0.25% in US Finn sheep, which was significantly lower than that in the NZ Finnish Landrace. The variant and genotype frequencies presented in Figure 8 for different breeds may not be comparable to other studies, because the numbers were typically small and the sheep typed were not necessarily representative of the breed as a whole. It should be noted that all the Perendale sheep in this study were homozygous and no sequence variation was detected in the *GDF9* region amplified.

The variant *A* was the most common variant detected in the NZ sheep breeds in this study. The frequencies of variant *B* (defined by the presence of c.994A, c.978G) in the flocks in this investigation were different to frequencies reported in other studies. For example, Kaczor (2017) reported a frequency of 17% for c.994A in Olkuska sheep, and Khodabakhshzadeh et al. (2016) reported that the frequency of c.994A was 63% in Kermani sheep. The Kermani sheep are thought to be the source of the c.994G>A variation. The maximum frequency observed for the c.994A variant in this study was 36% in the composite sheep from farms 3 and 4. Interestingly, in the current study, the c.994A variant was detected in all breeds except the Perendale sheep. A high frequency of c.994A was observed for the Merino and Polwarth sheep. The substitution c.994G>A has also been reported in the Afshari sheep breed (Eghbalsaied et al., 2017).

Phylogenetic relationships between species can be determined by comparison of DNA sequences (Hou, Pan, & He, 2014). Alignment of the DNA sequences obtained in this study with other reported sequences for *GDF9* revealed similarities of up to 98.3%. The similarity of the sequence with the presence of c.994A detected in this study is 99.2% similar to Brazilian Santa Ines sheep (GenBank Accession No. FJ429111.1) and Norwegian White sheep (GenBank Accession No. He866499.1).

Our sequence revealed the presence of three nucleotides sequence substitutions, c.994G>A, C.978G and c.1111A, while only the variants c.1111A and c.1034T were detected in the *GDF9* sequences obtained from Norwegian White sheep (Våge et al., 2013) and in Brazilian Santa Ines sheep (Silva et al., 2011) respectively. The sequences obtained from Norwegian White sheep were 99.7% similar to those from the Brazilian Santa Ines sheep, and the sequence of the *C* variant in the present study, was identical to the reported Norwegian White sheep DNA sequence (GenBank Accession No. He866499.1). There was also a high similarity (99%) between the sequence with the presence of

c.1111A and the Finnish Landrace sheep with the presence of nucleotide c.1034T (GenBank Accession No. NM_001142888.2).

When optimized, the PCR-SSCP procedure for typing *GDF9* detected the variant *A*, c.978A>G, c.994G>A and c.1111G>A substitutions for all the sheep studied. This technique allows large numbers of sheep to be typed rapidly, but the region amplified also encompassed other known substitutions including c.894A>C, c.974C>A, c.C943T, c.978A>G, c.994G>A, c.1034T>G, c.1040T>C , c.1042C>T, c.1111G>A, c.1124A>G, c.1184C>T, c.1203G>A and c.1219G>T, (Hanrahan et al., 2004; Nicol et al., 2009; Silva et al., 2011; Souza et al., 2001; Våge et al., 2013). Whether this nucleotide sequence variation did occur in the sheep studied here would be impossible to confirm without sequencing this region in all of the sheep. It is also conceivable that with further optimisation for gel temperature and running voltage, banding patterns may have varied, thus enabling other sequence variation to be identified (Sinville & Soper, 2007). This stated, PCR-SSCP can reliably detect single nucleotide changes in DNA sequences when used under optimised conditions (Bettinaglio et al., 2002). It also needs to be noted that Hanrahan et al. (2004) discovered eight variants (G1 to G8) of *GDF9* in Cambridge and Belclare sheep breeds using PCR-SSCP and sequencing. The DNA sequence of variant *A* (containing nucleotides c.978A, c.994G, and c.1111G), indicated that this variant exhibited 100% homology with the GenBank accession number sequence AF07854.2. In a study by (Hanrahan et al., 2004) the c.1184T variant of c.1184C>T (also known as FecGH: High Fertility) had effects on fertility phenotype. The c.1184T mutation causes sterility in homozygous ewes due to absence of the active form of the protein, but hyper-fertility and increased ovulation rates are observed in heterozygous ewes. Thc.1184C>T nucleotide sequence variation was not observed in the sheep typed in this study, but this is not unexpected as other studies have also not detected this variation (Paz et al., 2015; Vacca et al., 2010).

Although there is abundant research on the importance of variation in *GDF9* and fertility, a low frequency of c.994A and c.1111A was observed overall in this study for breeds of NZ sheep that nevertheless are quite fertile. It could therefore be concluded that these variants are not the only things responsible for increased fertility, and that other genes or environmental effects may be having greater impact on the fertility of these NZ breeds. Moreover, considering that some of the *GDF9* variation described above markedly increases fertility, one also needs to consider whether having excessively large litter-size is beneficial. After all, genetic variation like c.1184T, can lead to the production of lambs with low growth rates and that need hand-rearing (Abdoli, Zamani, Mirhoseini, Ghavi Hossein-Zadeh, & Nadri, 2016; Mullen & Hanrahan, 2014).

Generally, achieving genetic gain is difficult for fertility traits, because firstly it cannot be measured before maturity, and secondly because these traits are expressed in only one sex. Moreover, the

accurate measurement of the fertility traits can be difficult and expensive at the farm level. On the other hand, the high cost of genotyping limits its commercial use. For example, while it is claimed that Single Nucleotide Polymorphism (SNP) chips can be used to ascertain genotype (and thus phenotype), a large number of sheep are often required to train the chips, especially for low heritability traits, and accordingly the use of genomic selection with SNP chips is currently limited in sheep breeding programs. In contrast, the identification and use of single gene markers for key traits can be an appropriate and suitable method to improve production performance.

Together the results in this chapter provide valuable insights into the finding of three *GDF9* variants using the PCR-SSCP approach, and it justifies the further use of this approach for looking at more sheep and of other breeds to those studied here. Further studies on the effects of other *GDF9* SNPs (in introns or exons) could yield even more information allowing improvement of sheep fertility. Although no new mutations have been detected in this study but it is evident that *GDF9* is variable in NZ sheep. This lays foundation to further this type of analysis with more breeds from New Zealand, and elsewhere.

Chapter 3

Associations between the detected variations in *GDF9* and litter-size in New Zealand sheep

3.1 Introduction

Sheep farmers have an opportunity to improve the genetic merit of sheep through breeding, and how they choose to apply genetic information in making selection decisions. Research has identified useful information about sheep genetics and the use of 'DNA markers' to improve performance is becoming widespread. Specifically, animals with better performance for key production traits of economic significance, can be selected for use as breeding stock, and by both commercial farmers and sheep breeders. Fertility is one of the essential functional traits in sheep, and it is now well established that improving reproduction trait performance is feasible by accommodating the effects of genes that have been identified to affect reproductive performance. This has led to the development of improved breeding approaches, including the use of marker-assisted selection (MAS), where genotyping identifies genetic variation that marks desirable traits such as the number of lambs born per ewe, per year. Research has shown that MAS can assist improve reproduction traits, despite the heritability of these traits typically being low, and the fact that many of the reproduction traits are only expressed in one sex. Moreover, the approach has an added advantage, because fertility traits can be both difficult and expensive at the farm level.

In this context, the study of genes involved in fecundity has become of major interest to sheep science and farming. One of the most studied genes affecting sheep fertility is the Growth Differentiation Growth Factor 9 (*GDF9*) (Chung & Davis, 2014; Davis, 2005). This gene maps to ovine chromosome 5 (Sadighi et al., 2002), spans approximately 2.5 kilobases (kb) and contains two exons and a single 1126-base pair (bp) intron. The gene encodes a pre-propeptide of 453 amino acid residues, which produces an active mature peptide of 135 residues (Bodensteiner et al., 1999).

Nucleotide sequence variation has been described in *GDF9* by many researchers. For example, Hanrahan et al. (2004) described nine different alleles of *GDF9*, but among them only c.1184C>T had additive effects on prolificacy in Cambridge and Belclare sheep breeds. The c.1034T>G variation led to an increase in ovulation rate (82%) and prolificacy (58%) in Brazilian flocks (Silva et al., 2011), and variation reported by (Nicol et al., 2009) (c.1279A>C, also known as $FecG^{TT}$ or Thoka), results in an amino acid substitution of serine with arginine at position 427 (p.S427A). This increases ovulation rate in heterozygous individuals, but causes infertility in homozygous individuals. Nicol et al. (2009) also confirmed that c.1279C resulted in ~0.6 more lambs per ewe lambing in heterozygous animals.

Våge et al. (2013) investigated the effect of variation in *GDF9* in Norwegian White sheep. In this study, they identified that ewes homozygous for c.1111A produced more lambs when compared to heterozygous ewes, while the daughters of homozygous rams also produced more lambs (minimum 0.46 - 0.57 additional lambs). Mullen & Hanrahan (2014) revealed that c.1111A also affected litter-size in high prolificacy Finnish Landrace sheep in commercial flocks.

In the previous chapter, three variants and six unique genotypic banding patterns of the GDF9 gene were detected in some NZ sheep. It remains to be demonstrated if there is any significant association between the variants and litter size. There have been no studies on whether GDF9 variation occurs in New Zealand (NZ) sheep breeds, or whether it affects fertility traits. Accordingly, this Chapter aimed at testing the hypothesis that there are significant associations of GDF9 variation and its association with fertility was carried out in NZ Finnish Landrace, Finnish Landrace × Texel-cross and composite sheep.

3.2 Materials and methods

Ethics statement

This research project was carried out under license from the Animal Welfare Act 1999 (NZ Government) for research involving animals. And the collection of sheep blood drops by the nicking of their ears was covered by Section 7.5 Animal Identification, in: *Code of welfare: sheep and beef cattle (2016); a code of welfare issued under that act.* This process is considered to be a regular practice in farm management system, and cause little or no harm to animal, therefore no formal ethics review needed in this study.

Sheep studied

The litter-size data for ewes lambing in 2016 was obtained from one flock. The pedigree had a maximum depth of five consecutive generations. Sheep without records and unknown family history were omitted. A total of 241 ewes were analysed for this study. These included NZ Finnish Landrace sheep (n = 104), Finnish Landrace × Texel-cross sheep (n = 61), and composite sheep (farm 1) (of varying breed background, n = 76). These three groups were derived from a single large ewe flock farmed on pasture and all fed the same way in North Canterbury.

The blood samples analysed, DNA purification method, PCR amplification, SSCP analysis, genotyping and DNA sequencing were described in chapter two.

Statistical analyses

Analyses, undertaken in the R programme (Team, 2013), included determining the number of lambs born per ewe with different *GDF9* genotypes, and an analysis of variant and genotype frequencies in the Finnish Landrace, Finnish Landrace × Texel-cross and composite sheep.

Assessment of the presence or absence of each of the *GDF9* variants on fertility was conducted using an ASREML approach (Gilmour et al., 2015), and with both animal and sire models. The ASREML software was used to estimate the additive and dominance effects of single *GDF9* variants in the different models. In the models, the effect of each variant relative to other variants on litter-size was estimated. For example to estimate the additive effect of A relative to B and C, the genotypes were coded according to the 'number of copies' of *A*: *AA* = 2; *AB* or *AC* = 1; and *BB*, *BC* or *CC* = 0. For estimating the dominance effect of *A*, genotypes were coded according to the 'presence' of *A*: *AA* or *AB* or *AC* = 1; and *BB* or *BC* or *CC* = 0. The best complete model was selected by screening all possible subsets of the following full model:

1)$y_{ijkln} = \mu + \alpha_i + Breed_j + AGE_k + G_l + e_{ijkln}$

2) $y_{ijkln} = \mu + S_i + Breed_j + AGE_k + G_l + e_{ijkln}$

Where y_{ijkln} represents the phenotypic value of litter-size of the i^{th} ewe in 2016; μ is the average number of lambs born per ewe; G_l is the additive effect of *GDF9* variant, $Breed_j$ is the fixed effect of breed (when the three breeds were analysed together); AGE_k is the ewe's age at lambing fitted as covariate; αi is the random animal effect of ewe i (~N(0, σ^2_aa) when full pedigree matrix A was fitted in the animal model; S_i is the random effect of sire of ewe i ~N(0, σ2s) when the relationships between the sires in Matrix S was fitted in sire model; and e_{ijkln} is the random residual effect for each observation (~N(0, σ2e)).

3.3 Results

As shown in chapter two, the SSCP analysis revealed three banding patterns (named variants *A*, *B* and *C*), and six genotypes of these banding patterns (*AA, AB, AC, BB, BC,* and *CC*) in all three groups of sheep. The sequencing of homozygous genotypes confirmed that the three variants were unique DNA sequences, and upon comparison of these sequences, three nucleotide variations c.978A>G, c.994G>A and c.1111G>A were identified in the fragment of *GDF9* that was amplified. The variant *B* was defined by the presence of nucleotides c. 978G and c.994A and variant *C* contained c.1111A.

The variant frequency distribution data indicated a predominance of *A* in the sheep typed. While all three variants were detected in the three different groups of sheep, some genotypes were not

identified. The frequency of variant *B* (c.978G, c.994A) in the Finnish Landrace, Finnish Landrace x Texel-cross and composite sheep was 0.01, 0.01, and 0.17 respectively, while that of c.1111A was 0.08, 0.18, and 0.03 respectively. No homozygous c.994A individuals were detected for the Finnish Landrace and Finnish Landrace × Texel-cross sheep, while the genotype frequency was very low for the composite sheep (0.05). A low frequency of homozygous c.1111A ewes was observed for the Finnish Landrace x Texel- cross sheep, while the frequency of heterozygous ewes was 0.32. With the composite sheep, the c.1111A variant was present at a low frequency (0.3), while in these sheep c.994A was present at a frequency of 0.17.

The average litter size for the studied group was 2.47±1.04 in Finnish Landrace , 1.92±0.66 in Finnish Landrace × Texel-cross sheep, and 1.91±0.49 in composite sheep= (All groups=2.18±0.85).

In many investigations, only the additive effects of putative casual mutations on variation in traits are studied, but estimation of the dominance effects are beneficial when using terminal-sire breeding systems. The association results from this study are shown in Tables 5. The association results are shown in Tables 5 (additive effect and (dominance effect). ewe age affected fertility in the Finnish Landrace and composite sheep, but not the Finnish Landrace × Texel-cross sheep. They indicate associations between the variation in *GDF9* and litter-size, and suggest additive and dominance effects respectively. In the Table 5, there was evidence for the Finnish Landrace × Texel-cross sheep, of an association ($P < 0.05$) between c.1111A (versus c.1111G) and litter-size, while for the Finnish Landrace (0.33 ± 0.292; P = 0.270) and composite sheep (-0.43 ± 0.316; P = 0.127), no association was observed. The effect of the GDF9 gene variation appeared to be additive, with one copy of c.1111A increasing litter size by 0.43, and two copies by 0.86 in the Finnish Landrace × Texel-cross ewes. The effect of c.1111A was 0.34 ± 0.15 (P = 0.027) compared to those ewes with c.1111G using an animal model, when all groups were analysed together (i.e. the effect of breed was included in the model). Litter-size appeared to be unaffected by both variant B and variant A in all the groups when GDF9 variant was fitted as an additive effect (Table 5). Table 5 reveals the estimated effect of the GDF9 variants and nucleotide substitutions when fitted as having a dominance effect. Once again, ewe age affected fertility in the Finnish Landrace and composite sheep, but not the Finnish Landrace × Texel-cross sheep. There was evidence, for the Finnish Landrace × Texel-cross sheep, of an association (0.47 ± 0.222; P = 0.037) between c.1111A and litter-size, but this was not observed with the Finnish Landrace sheep (0.33 ± 0.296; P = 0.270), or the composite sheep (-0.43 ± 0.316; P = 0.172). There was an overall effect of c.1111A on litter-size (0.35 ± 0.162; P = 0.033), but no effects were observed for variant *B* or variant *A*.

Table 5 Estimated effect of *GDF9* variants and nucleotide substitutions fitted as having an additive and dominance effects on number of lambs born per ewe in three groups of NZ sheep

Groups	Models	Type of effect	*GDF9* Variation	P-value	Effect (± se)[a]	Source of variation	
						Group	Ewe age
All Groups	Animal	Additive	*A*	0.227	-0.13 ± 0.114	**0.019**	**0.001**
	Animal	Additive	*B*	0.536	-0.10 ± 0.164	**0.027**	**0.001**
	Animal	Additive	*C*	**0.027**	0.34 ± 0.154	**0.022**	**0.001**
	Animal	Dominance	*A*	0.714	-0.11 ± 0.331	**0.022**	**0.001**
	Animal	Dominance	*B*	0.64	-0.09 ± 0.200	**0.028**	**0.001**
	Animal	Dominance	*C*	**0.033**	0.35 ± 0.162	**0.022**	**0.001**
	Sire	Additive	*A*	0.271	-0.12 ± 0.112	**0.019**	**0.001**
	Sire	Additive	*B*	0.472	-0.11 ± 0.162	**0.027**	**0.001**
	Sire	Additive	*C*	**0.029**	0.33 ± 0.152	**0.022**	**0.001**
	Sire	Dominance	*A*	0.812	-0.07 ± 0.33	**0.022**	**0.001**
	Sire	Dominance	*B*	0.593	-0.1 ± 0.198	**0.028**	**0.001**
	Sire	Dominance	*C*	**0.034**	0.34 ± 0.159	**0.022**	**0.001**
Finnish Landrace	Animal	Additive	*A*	0.172	-0.36 ± 0.263	-	**0.001**
	Animal	Additive	*B*	0.328	0.66 ± 0.67	-	**0.001**
	Animal	Additive	*C*	0.27	0.33 ± 0.296	-	**0.001**
	Animal	Dominance	*A*	0.909	-0.09 ± 0.906	-	**0.001**
	Animal	Dominance	*B*	0.328	0.66 ± 0.676	-	**0.001**
	Animal	Dominance	*C*	0.27	0.33 ± 0.296	-	**0.001**
	Sire	Additive	*A*	0.147	-0.37 ± 0.251	-	**0.001**
	Sire	Additive	*B*	0.236	0.75 ± 0.627	-	**0.001**
	Sire	Additive	*C*	0.26	0.33 ± 0.292	-	**0.001**
	Sire	Dominance	*A*	0.614	-0.44 ± 0.893	-	**0.001**
	Sire	Dominance	*B*	0.236	0.75 ± 0.627	-	**0.001**
	Sire	Dominance	*C*	0.26	0.33 ± 0.292	-	**0.001**
Finnish Landrace x Texel cross	Animal	Additive	*A*	0.135	-0.30 ± 0.199	-	0.261
	Animal	Additive	*B*	0.07	-1.33 ± 0.717	-	0.250
	Animal	Additive	*C*	**0.036**	0.43 ± 0.202	-	0.239
	Animal	Dominance	*A*	0.521	-0.46 ± 0.713	-	0.270
	Animal	Dominance	*B*	0.07	-1.33 ± 0.717	-	0.250
	Animal	Dominance	*C*	**0.037**	0.47 ± 0.222	-	0.240
	Sire	Additive	*A*	0.135	-0.30 ± 0.199	-	0.261
	Sire	Additive	*B*	0.07	-1.33 ± 0.717	-	0.250
	Sire	Additive	*C*	**0.036**	0.43 ± 0.202	-	0.239
	Sire	Dominance	*A*	0.522	-0.46 ± 0.714	-	0.270
	Sire	Dominance	*B*	0.07	-1.33 ± 0.717	-	0.250
	Sire	Dominance	*C*	**0.037**	0.47 ± 0.222	-	0.240
Composite sheep	Animal	Additive	*A*	0.551	0.06 ± 0.112	-	**0.002**
	Animal	Additive	*B*	0.898	-0.01 ± 0.116	-	**0.002**
	Animal	Additive	*C*	0.172	-0.43 ± 0.316	-	**0.002**
	Animal	Dominance	*A*	0.963	-0.01 ± 0.278	-	**0.002**
	Animal	Dominance	*B*	0.892	0.02 ± 0.147	-	**0.002**
	Animal	Dominance	*C*	0.172	-0.43 ± 0.316	-	**0.002**
	Sire	Additive	*A*	0.551	0.06 ± 0.112	-	**0.002**
	Sire	Additive	*B*	0.899	-0.01 ± 0.116	-	**0.002**
	Sire	Additive	*C*	0.157	-0.45 ± 0.315	-	**0.002**
	Sire	Dominance	*A*	0.954	-0.01 ± 0.278	-	**0.002**
	Sire	Dominance	*B*	0.893	0.02 ± 0.147	-	**0.002**
	Sire	Dominance	*C*	0.157	-0.45 ± 0.315	-	**0.002**

[a]Estimation of the effect +/- standard error of each variant relative to other variants on litter size. *P* < 0.05 in bold type

It is apparent from the Table 5 that all the effects are significant in different models except ewe age in the model for Finnish x Texel-cross sheep (P > 0.05). Thus, ewe age appears to have an influence on the estimate of the effect of the variations on litter-size.

3.4 Discussion

One of the most important goals in sheep breeding is finding functional variations that affect highly favourable traits such as fertility. These affect overall profitability in the sheep industry. Detecting such variations potentially leads to the design of efficient breeding programmes, especially those that can use MAS and thus increases the accuracy of selection in farm animals, more rapidly and in a cost effective manner. It is of great advantage if a trait or traits are only expressed upon maturity, as is the case with reproduction. Against this background, this study reflected the broad interest in the role of *GDF9* in controlling sheep fertility, through its activity in controlling ovarian function.

Together the results in this study provide valuable insights into variation in *GDF9* revealed using the PCR-SSCP approach. Three nucleotide sequence variations (c.978A>G, c.994G>A and c.1111G>A) were detected in Finnish Landrace sheep, Finnish Landrace x Texel-cross sheep and composite sheep. The modelling approach employed suggested that the presence of *GDF9* variant C, which contains c.1111A, is associated with litter-size in the Finnish Landrace x Texel-cross sheep, whereas associations with litter-size were not observed for the other variants (*A* contains c.978A, c.994G, and 1111G and *B*, which contain c.978A>G and c.994G>A respectively).

The association between the number of lamb births per ewe and genotypes was tested. When analysis was conducted across all breeds, the c.1111A variant was associated with litter-size in Finnish Landrace x Texel-cross. However, no significant associations were found between this nucleotide substitution and litter size in the two other groups of sheep investigated in this study. There was no association in the Finnish Landrace breed and composite sheep which might be attributable to the phenotypic expression of one allele that is somewhat dependent on other alleles, especially if there are multiple interacting mutations. Therefore, the phenotypic effect of any given allele may be observed in one breed, while being absent in another (Abdoli et al., 2016). It is also possible, given the low frequency of c.1111A in the sheep studied, that other phenotypic effects masked or diluted the association.

The data were analysed using both animal and sire models and both dominance and additive effects were fitted in the models. All the fixed effects fitted in the models were significant except for the age at parturition in the model for Finnish x Texel-cross ewes, probably because most of the Finnish x Texel-cross ewes in the model were at a similar age at parturition. Unexpectedly, in the current study the estimation of variant effect on litter-size as an additive effect, bore a close resemblance to a dominance effects. A reasonable explanation for this is that the standard errors of the estimation of effects were very high; or perhaps the study had a low number of either heterozygous or homozygous ewes. For example, the effect of c.1111A on litter-size in the Finnish Landrace x Texel-cross was 0.43 and 0.47 in both the additive and dominance effects respectively. The only reason for

this is that only one homozygous (*CC*) sheep was detected in the sheep studied. Although there was no significant difference between the results obtained from the animal model and the sire model, the animal model was preferred to the sire model in this study because the animal model uses the relationship between all animals in the pedigree to measure the polygenic effects, and it can better separate phenotypic variance into additive genetic and residual effects components (Henderson & Quaas, 1976).

Previous studies have demonstrated that genetic variation in *GDF9* was associated with increased ovulation rates and litter-size in sheep. The sequence variation c.1111A detected in the current study had an effect on litter-size that is consistent with the findings of Våge et al. (2013) who found a strong association between this substitution and litter-size in Norwegian white sheep. They demonstrated that daughters of rams that were homozygous for c.1111A gave birth to 0.46 to 0.57 additional lambs, while daughters of rams heterozygous for c.1111A gave birth to 0.20 to 0.25 additional lambs. Kaczor (2017) found that Olkuska ewes with one copy of the c.1111G>A substitution, had an increase in litter-size of 0.55. The lambs from homozygous ewes were twice the size of the heterozygous ewes.

In the present study, we found that Finnish Landrace × Texel-cross ewes with one copy of c.1111A, produced approximately 0.43 more lambs, than the c.1111G homozygous ewes. These results are consistent with the findings of (Mullen & Hanrahan, 2014) who reported no statistically significant effect of a single copy of c.1111A on ovulation rate in Finnish Landrace ewes. Although the Finnish Landrace sheep studied here also failed to reveal an association between fertility litter-size and a single copy of c.1111A. It needs to be noted that the average litter-size for the Finnish Landrace sheep (2.4) was larger than for the Finnish Landrace × Texel-cross sheep and composite sheep (1.8) in this study. This might suggest that the effect of c.1111A was not found to affect litter-size in the pure Finnish Landrace sheep because other genes were also affecting their fertility, and the effect of c.1111A was small in comparison to these other genes. In contrast in Belclare sheep, Hanrahan et al. (2004) noted an association between c.1111A and ovulation rate. One copy of this variant increased ovulation rate by +0.17 in heterozygous ewes, compared to wild-type ewes without the mutation. The effect was non-additive though.

No mutation in *GDF9* with consistent major effect on litter size across breeds was detected in the current study. Hanrahan et al. (2004) did not detect any association of c.1111A with litter-size in Belclare and Cambridge sheep, probably because of the infrequency of this mutation in the sheep they studied. This contrasts the finding obtained in this study where c.1111A is associated with litter-size in Finnish Landrace x Texel-cross sheep, but is consistent with the findings of Hanrahan et al. (2004), who found that none of the sequence variation detected in the current study had additive effects on prolificacy.

The other *GDF9* nucleotide sequence variations detected in this study (c.994G>A and c.978A>G) were also reported by Kaczor (2017), who illustrated that ewes carrying c.C994A had a decrease of 0.18 in lamb litter-size. The current study was unable to establish an association between these variants and litter-size.

Undiscovered sequence variation may exist in other regions of the GDF9 gene and this may also affect the activity of the gene and the associations that are observed with some of the previously described nucleotide sequence variations. Equally, while the results of this study reflect those of Hanrahan et al. (2004), who did not observe an association between the variant *B* (c.994G>A/c.978A>G) and litter-size, it may also be because of the low frequency of this sequence variation in the Finnish Landrace sheep and Finnish Landrace x Texel-cross sheep that were studied. It should also be noted that while the analysis of litter-size failed to identify any significant difference, the estimate for the effect of a single copy of c.994A on litter-size was - 1.33 ± 0.717 (P = 0.070) in the Finnish Landrace x Texel-cross sheep and 0.66 ± 0.676 (P = 0.328) in the Finnish Landrace sheep, with the former suggesting that a trend may exist. This therefore requires further investigation in sheep carrying these sequence variations. The findings in this chapter also differ from those of Bravo et al. (2016), who revealed that both c.994A and c.978G as detected variant *B* in this study were associated with an increase in with litter-size.

The GDF9 variation detected in this study could be one, but not the sole factor in determining litter-size in the sheep studied. As described previously, litter-size is affected by many things including the management (e.g. nutrition) of sheep and other environmental factors. The interaction between genotype and environment may also play a role in the number of lambs born per ewe per year; therefore, it is vital to consider the essential issue of environmental factors in sheep breeding programmes. Even so, the results of research like the current study could be applied in marker-assisted selection programmes, but it can be concluded that these variants are probably not the only ones responsible for the higher fertility in the sheep studied, and that other variation in *GDF9* and other genes may also be involved.

This study has gone some way towards enhancing our understanding of how establishing the association between functional variations in the *GDF9* and sheep fertility can be done quickly and cost-effectively using a PCR-SSCP approach, and the technique would certainly have utility in investigating other sheep breeds and their fertility. This study lays a strong foundation to further this type of analysis with more common New Zealand breeds, not least the main maternal breeds, the Romney, Perendale, and Coopworth. The presence of the functional variation confirms that further research should be undertaken to detect more mutations associated with litter-size, and on a broader scale on some other candidate genes (such as *BMP15* and *BMPR1B*). Using *GDF9* variation as a genetic marker in a multi-

gene pyramiding approach could provide a way to improve litter-size and hasten the breeding of highly prolific sheep.

Chapter 4

Identification of a Single Codon Deletion in the bone morphogenetic protein 15 (*BMP15*) gene in New Zealand sheep

4.1 Introduction

Kosgey, van Arendonk, and Baker (2003) highlighted that litter-size and lambing frequency are essential traits in sheep breeding, and that effective evaluation of these functional traits underpins genetic improvement plans. As described in earlier chapters, several genes, proteins, and hormones are involved in the regulation of growth and reproductive performance (Chu et al., 2007; Davis et al., 2001; Galloway et al., 2000).

In this thesis, three genes, growth differentiation factor 9 (*GDF9*), bone morphogenetic protein 15 (*BMP15*), and bone morphogenetic protein receptor 1B (*BMPRIB*), known to affect reproductive performance were investigated in New Zealand (NZ) sheep. . Of these genes, BMP15 produces a protein (BMP15) that causes increased sensitivity of ovarian granulosa cells to follicular stimulating hormone (FSH), therefore speeding up follicular development and precocious ovulation of small follicles in heterozygous ewes (Moore & Shimasaki, 2005). The BMP15 protein belongs to the transforming growth factor ß (TGF-ß) superfamily TGFβ superfamily, and BMP15 is a gene of 5.4 kb in length that consists of two exons separated by one intron (exon 1 (accession number AF236078.1) and exon 2 (accession number AF236079.1). The gene is located on ovine chromosome X (50970938-50977454 bp, OARv3.1) and is associated with 10 variations and with two exons and seven domain annotations and features (Abdoli et al., 2016).

The BMP15 gene is known to contain nucleotide sequence variation, some of which affect sheep fertility, and these include, c.1279A>C (Nicol et al., 2009), c.950C>T (Demars et al., 2013), c.1009A>C (Kaczor, 2017), c.897A>T (Davis et al., 2001; Galloway et al., 2000), Woodlands (FecX2; Davis, 2005), c.963G>A (Bodin et al., 2007; Drouilhet et al., 2009), c.487_503del (Martinez-Royo et al., 2008), c.873C>T, c.718C>T and c.1100T>G (Galloway et al., 2000; Hanrahan et al., 2004; Montgomery et al., 2001).

It has been reported that heterozygous mutations in *BMP15* lead to an increase in ovulation rate, and litter-size, while homozygous ewes are sterile (Chu et al., 2007). Hanrahan et al. (2004) identified four nucleotide sequence variations in Cambridge and Belclare sheep, and that one of them (c.31_33del) eliminated a single Leucine residue (p.Leu11del), but appeared to have no phenotypic effect. Similar results previously described this deletion (without any phenotypic effect) were obtained

by (Galloway et al., 2000). In contrast to this, (Guo et al., 2004) detected c.31_33del in Small Tail Han sheep, and it was associated with decreased litter-size in this Chinese breed. Monteagudo et al. (2009) reported increased litter-size in Rasa Aragonesa sheep associated with a 17 bp deletion in *BMP15*, while (Zamani et al., 2015) observed a point mutation (c.971A>G) in exon 2 of *BMP15*, which was found to be associated with prolificacy in Iranian Mehraban and Lori sheep.

All these studies have established that the BMP15 gene plays a crucial role in sheep fertility, but little is known about variation in this gene in NZ sheep and whether variation, if present, affects litter-size. Accordingly this chapter investigated *BMP15* in different NZ sheep breeds.

4.2 Materials and methods

Sample collection and DNA purification

This research project was carried out in accordance with the Animal Welfare Act 1999 (New Zealand Government) for research involving animals. And the collection of sheep blood drops by the nicking of their ears was covered by Section 7.5 Animal Identification, in: *Code of welfare: sheep and beef cattle (2016); a code of welfare issued under that act*. This process is considered to be a regular practice in farm management system, and cause little or no harm to animal, therefore no formal ethics review needed in this study.

In the present study, a total number of eight hundred and fifty two sheep from fifteen different NZ sheep breeds and a composite sheep were investigated. These included: Finnish Landrace (n = 148), Finnish Landrace × Texel-cross (n = 45), composite sheep are sheep bred from a wide variety of genetic backgrounds based on selection for key production traits. Their background is typically very diverse, and in the case of the sheep described here will include at very least NZ Romney, Texel, East Friesian and Finnish Landrace sheep based on what is known about the history of the flock (n = 59), White Dorper (n = 71), Perendale (n= 48), Merino (n = 80), Romney (n = 90), Texel (n = 28), Corriedale (n = 43), Wiltshire (n = 48), Coopworth (n = 48), Easycare (n = 24), Lleyn (n = 24), Shropshire (n = 24), Southdown (n = 24) and Dohne (n = 48). The samples were collected from different farms across New Zealand.

In this study, FTA^{TM} cards (Whatman BioScience, Middlesex, UK) were used for blood collection from a small incision in the ear of the sheep. DNA was extracted from the blood samples by punching a 1.2-mm disc from the FTA card, followed by genomic DNA purification using a two-step procedure described by Zhou et al. (2006). To begin this process, the FTA card punch was placed in tubes containing 200 µL of 20 mM NaOH, left for 20 to 30 minutes at 60 °C, or until the disk became white. All the liquid was then removed and the disk equilibrated in 200 µL of 1× TE buffer (10 mM Tris– HCl,

0.1 mM EDTA, pH 8.0). After this washing and equilibration, the liquid was again removed, and the disks were left to air dry in the tubes overnight.

PCR amplification and SSCP analysis

Polymerase Chain Reaction - Single Strand Conformation Polymorphism (PCR-SSCP) analyses were used to search for sequence variation in 480 bp and 310 bp fragments of exon 1 and 2 of BMP15, respectively. Primers were designed based on GenBank sequence NC_019484.2 to amplify regions that encompassed nucleotide variation reported previously to have associations with litter- size. These included c.31_33del (Gua et al., 2004), c.302_304del, c.301G >T (Lassoued et al., 2017), and in exon 1; and c.873C>T (Galloway et al., 2000), c.897A>T (Galloway et al., 2000; Davis et al., 2005), c.963G>A (Bodin et al., 2007), c.950C>T, and c.1009A>C (Demars et al., 2013) in exon 2. The primers were 5'- CCTTGCCCTATCCTTTGTG -3' (forward) and 5'-CCTCCCACCAGAACAATA-3' (reverse) for a 5'-UTR/exon 1/intron 1 region and 5'-GCAGGCAGTATTGCATCGGAAG-3' (forward) and 5'-CCTCAATCAGAAGGATGCTAATGG -3' (reverse) for an exon 2 region of BMP15. The PCR amplifications were performed in a 15-µL reaction containing the genomic DNA on one 1.2-mm punch of FTA card, 0.25 µM of each primer, 150 µM of each dNTP (Bioline, London, UK), 0.3 mM Mg2+, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1× reaction buffer supplied with the enzyme. For both regions, the amplification were undertaken as follows: initial denaturation at 94 °C for 2 minutes, followed by 36 cycles of 94 °C for 30 seconds (denaturation), 59 °C for 30 seconds (annealing), and 72 °C for 30 seconds (elongation); with a final extension step at 72 °C for 5 minutes.

For the SSCP analysis, a 0.7-µL aliquot of each amplicon was mixed with 7 µL of loading dye (98% Formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol), and after denaturation at 95 ^{o}C for 5 minutes, the samples were rapidly cooled on wet ice and immediately loaded on to 16 cm × 18 cm, 12% acrylamide: bisacrylamide (37.5:1; Bio-Rad, Hercules, CA, USA) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad), at 350 V for 18 hours at 7 ^{o}C in 0.5x TBE buffer. The DNA fragments were visualized using a silver nitrate staining method (Byun et al., 2009). Briefly, the gels were bathed in a solution of 10% ethanol, 0.5% acetic acid and 0.2% $AgNO_3$ for 10 minutes. Next, the gels were rinsed with distilled water then developed with a solution of 3% NaOH and 0.1% HCOH until dark-staining bands appeared on the yellow background of the gel.

Sequencing of variants and sequence analyses

PCR amplicons representing different banding patterns from sheep that appeared to be homozygous were sequenced for two samples in both directions at the Lincoln University DNA Sequencing Facility to confirm that variants detected represented unique sequences. Variants that were only found in heterozygous sheep were sequenced using an approach described by (Gong et al., 2011). Briefly, a

band corresponding to each variant was excised as a gel slice from the polyacrylamide gel, macerated, and then used as a template for re-amplification with the original primers. This second amplicon was then sequenced. Sequence alignments, translations, and phylogenetic analysis were carried out using DNAMAN (version 5.2.10, Lynnon BioSoft, Vaudreuil, Canada).

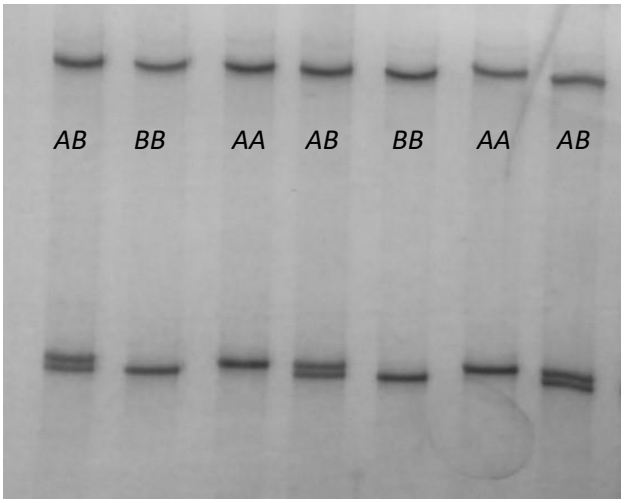
Statistical analysis

Allele and genotype frequencies were calculated in R programming software (Team, 2013)),for White Dorper, Finnish Landrace sheep, the Finnish Landrace × Texel-cross, Perendale, Merino, Romney, Corriedale, Wiltshire, Dohne, Coopworth, Easycare, Southdown, Shropshire, Lleyn and composite sheep from different sheep farms.

4.3 Results

A PCR-SSCP analysis of the 480 bp amplicon of *BMP15* exon 1 in the different sheep breeds, revealed two banding patterns (named *A, B*), and three genotypes of these banding patterns (*AA, AB, BB*) (Figure 9). Sequencing confirmed that the two variants were unique DNA sequences and a three base pair deletion (c.31_33del) was detected. The c.31_33del has been reported previously (Hanrahan et al., 2004).

No variation was observed in the 310 bp amplicon of *BMP15* exon 2, as revealed by PCR-SSCP.



(A)

Variation	*A*	*B*
c.31_33del	CTT	-

(B)

Figure 9 a) The resulting gel patterns from polymerase chain reaction single-strand conformational polymorphism (PCR-SSCP) analyses indicating genotypes *AA*, *AB*, and *BB*. (b) The sequence variation detected in the exon 1 region for *BMP15* in the NZ sheep breeds.

Figure 10 presents the exon 1 nucleotide sequences of *BMP15*, including the c.31_33del. This would result in the deletion of a single leucine residue (p.Leu20del). The nucleotide sequence is deposited in GenBank with the accession number AF236079).

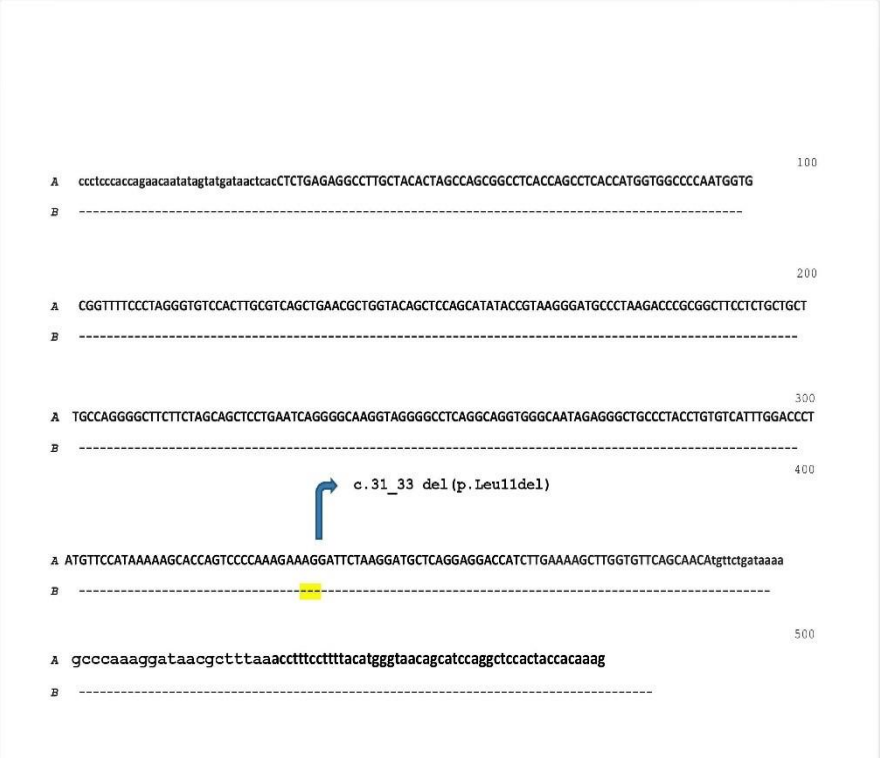


Figure 10 Nucleotide sequences of bone morphogenetic protein 15 (*BMP15*) in exon 1 variants *A* and *B*.

Nucleotides in the coding region are shown in uppercase, while those outside the coding region are in lowercase. The c.31_33del is shown above the sequences.

Table 6 presents an overview of the presence of c.31_33del in different New Zealand sheep breeds. The c.31_33del appears to be common in New Zealand sheep. The deletion c.31_33del is present in all breeds except Easycare, Shropshire and Southdown, but no homozygous BB sheep were detected in the Wiltshire, Finnish Landrace x Texel-cross, and no heterozygotes nor homozygotes in Easycare, Shropshire, and Southdown sheep.

Table 6 Allelic and genotype frequency of variants of *BMP15* exon 1 in sixteen New Zealand sheep groups/breeds

		Frequency		Genotype frequency[#]		
Group	**Number**	**Variant *A***	**Variant *B***	**Homozygous (*AA*)**	**Heterozygous (*AB*)**	**Homozygous (*BB*)**
White Dorper	71	0.31	0.69	0.20	0.23	0.58
Perendale	48	0.88	0.13	0.88	0.00	0.13
Finnish Landrace	148	0.77	0.23	0.64	0.26	0.09
Merino	80	0.58	0.42	0.50	0.16	0.34
Romney	90	0.81	0.19	0.78	0.06	0.17
Texel	28	0.89	0.11	0.89	0.00	0.11
Corriedale	68	0.54	0.46	0.54	0.00	0.46
Finnish Landrace ×Texel-cross	45	0.92	0.08	0.84	0.16	0.00
Composite sheep	59	0.68	0.32	0.46	0.44	0.10
Wiltshire	48	0.75	0.25	0.50	0.50	0.00
Coopworth	48	0.92	0.08	0.92	0.00	0.08
Dohne	48	0.63	0.38	0.63	0.00	0.38
Easycare	24	1	0	1	0	0
Lleyn	24	0.71	0.29	0.71	0	0.29
Shropshire	24	1	0	1	0	0
Southdown	24	1	0	1	0	0

One interesting finding in the table is that for a number of breeds investigated in this study there are many sheep that are apparently homozygous for the *A* and *B* variants (*AA* and *BB*) including, Perendale, Texel, Corriedale, Coopworth, and Dohne sheep breeds, but only a few or no heterozygous *AB* sheep are detected. The lack of heterozygous genotypes while the minor allele frequency is high likely reflects the fact that *BMP15* is located on the X chromosome, and hence what appears to be homozygosity is actually hemizygosity (i.e. the genotypes sheep were mainly rams). Variant *A* would appear to be the most common in most of the sheep, but as can be seen from Table 6, the frequency of variant *B* (0.69) was more than variant *A* (0.31) in White Dorper sheep. A high frequency of variant *A* was detected in Easycare, Shropshire and Southdown sheep (1), whereas a high frequency of variant *B* (c.31_33del) was detected in White Dorper and Corriedale, 0.69 and 0.46 respectively.

Heterozygous composite sheep were found at a frequency of 44%, but only 6% of the NZ Romney sheep were heterozygotes. The frequency of heterozygous sheep was similar in the Finnish Landrace x Texel-cross sheep and Merino sheep (16%). The highest frequency of homozygous sheep with the Leu codon deletion was observed for White Dorper samples (58%), and the lowest frequency was for Coopworth sheep (0.08%). All sampled Finnish Landrace x Texel-cross, Easycare, Shropshire and Southdown sheep in our study were homozygous for variant *A* (without the Leucine codon deletion). The frequency of variant *A* was very high in Coopworth and Texel sheep as well (0.92 and 0.89 respectively).

4.4 Discussion

The introduction and development of a commercial DNA test (called the Inverdale gene test) for the c.897A>T (p.Val299Asp) BMP15 gene variation by AgResearch in New Zealand, led to an increase in the use of a specific *BMP15* mutation in flocks in New Zealand, Australia, and Scotland. It is now well established that homozygous ewes are infertile, and thus commercial breeders must avoid mating two carrier parents (Davis et al., 2005).

Without BMP15 being present, oocytes continue to grow in the absence of granulosa cell proliferation until they are unable to be supported by the residual granulosa cells, and then they degenerate (Braw-Tal et al., 1993; Smith et al., 1997). Although the absence of functional BMP15 blocks follicular growth in homozygous mutant sheep, inactivation of only one copy of BMP15 has been reported to increase ovulation rate (Davis et al., 1991; Davis et al., 1992). In heterozygous sheep, the reduction in expression of active BMP15 may reduce the number of mitotic divisions in the granulosa cells, which causes a reduction in the amount of steroid and inhibin release by each follicle. This process, in turn, can cause a delay in the suppressive effects on plasma FSH, resulting in more follicles being prepared for ovulation. The reduction in BMP15 also appears to increase the sensitivity of follicles to FSH, which accelerates follicle growth (Montgomery et al., 2001).

The c.897A>T (p.Val299Asp) BMP15 gene variation was not observed in the sheep studied here. The only variation found was a previously reported three base pair deletion (c.31_33del, p.Leu11del) in *BMP15* exon 1, with three different genotypes (*AA*, *AB*, and *BB*) being revealed using a PCR-SSCP typing approach. Variant *B* contained the c.31_33del (p.Leu11del) deletion.

These are similar findings to the results obtained with Chinese Small Tail Han Sheep (Guo et al., 2004). These authors reported the same deletion (p.Leu11del) in the signal sequence of *BMP15* and didn't identify any other variation in the Han sheep. They reported frequencies for their *A* variant (without c.31_33del) and *B* (c.31_33del) of 0.73 and 0.27 respectively. This approximately matches the variant frequencies observed in the Finnish Landrace and Wiltshire sheep in this study, although the

frequencies are confounded by the gender of the sheep not being known for the sheep typed. The presence of c.31_33del reflects similar findings in other New Zealand sheep (Galloway et al., 2000; Hanrahan et al., 2004), but with both studies failing to detect any association between this sequence variation and litter-size.

A low frequency of heterozygous genotypes was detected in the screened animals. This was probably as a result of male lambs appearing to be homozygous, when they were actually hemizygous for a single variant of *BMP15*. This stated, the absence of the *B* variant in the Easycare, Shropshire and Southdown sheep, may suggest the deletion is not present in those breeds, but this result would need to be confirmed by typing many more sheep of this type/breed.

Equally, only a small portion of the BMP15 gene was studied in this chapter. Before it should be accepted that the only variation in *BMP15* observed in the breeds/types of sheep studied is the presence of the c.31_33del in exon 1, the rest of the BMP15 gene, including upstream and downstream nucleotide sequences also need to be characterised in detail. In this respect no sequence variation was found in exon 2 of the sheep, and regardless of their apparent variation in prolificacy. This finding contrasts other studies which have detected variation in exon 2 of *BMP15* in other the breeds including Iranian sheep (Amini et al., 2018) Grivette and the Olkuska sheep (Demars et al., 2013). Given the variation in prolificacy between the sheep breeds, we did not identify any variation in exon 2. It is likely that only c.31-33del in exon 1 observed in BMP15 gene in our studied sheep was a mutation affecting prolificacy over so many decades of evolutionary selection, but further studies using larger sample sizes are needed to confirm these results.

Chapter 5

Associations between the detected variation in *BMP15* and litter-size in New Zealand sheep

5.1 Introduction

Genetic variation in *BMP15* that results in changes in amino acid sequence contributes to variation in prolificacy in sheep, and variants with a known functional effect have been found to be associated with increased litter-sizes in sheep (Bodin et al., 2007; Davis, 2005; Demars et al., 2013; Guo et al., 2004; Hanrahan et al., 2004; Martinez-Royo et al., 2008; Zamani, Nadri, et al., 2015).

The BMP15 protein is a strong stimulator of granulosa cell mitosis and proliferation (Otsuka et al., 2000) and also the mRNA expression of granulosa cell kit ligand, a factor which is necessary for early follicle growth (Otsuka & Shimasaki, 2002). The BMP15 protein has an important role in developing early follicle growth since it has no effect on FSH-induced oestradiol synthesis (Moore, et al., 2004). Otsuka, et al (2001) found that BMP15 suppresses mRNA expression of the FSH receptor, which results in inhibition of FSH-dependent progesterone synthesis. The BMP15 gene (BMP15) is known to contain nucleotide sequence variation, some of which affects sheep fertility. This includes: c.897A>T (Davis et al., 2001; Galloway et al., 2000), c.873C>T, c.718C>T and c.1100T>G (Galloway et al., 2000, Hanrahan et al., 2004; Montgomery et al., 2001), c.31_33del (albeit named differently: Galloway et al., 2000; Hanrahan et al., 2004; Guo et al., 2004), c.963G>A (Bodin et al., 2007; Drouilhet et al., 2009), c.487_503del (Martinez-Royo et al., 2008), c.1279A>C (Nicol et al., 2009), c.950C>T and c.1009A>C (Demars et al., 2013), and c.755T>C (Amini et al., 2018).

It has been suggested that heterozygous mutations in BMP15 lead to an increase in ovulation rate, and litter- size, while homozygous ewes are sterile (Chu et al., 2007). Hanrahan et al. (2004) identified four nucleotide sequence variations in Cambridge and Belclare sheep, and that one of them (c.31_33del; but named differently in that paper) eliminated a single leucine residue (p.Leu11del), but appeared to have no phenotypic effect. Similar results were obtained by Galloway et al. (2000). In contrast, Guo et al. (2004) demonstrated that this three base pair deletion (c.31_33del) was associated with fertility in Small Tail Han sheep (with ewes that didn't have the deletion having greater fertility than those ewes that had the deletion). Additionally, Monteagudo et al. (2009) reported increased litter- size in Spanish Rasa Aragonesa sheep associated with a 17 bp deletion in BMP15, while Zamani et al. (2015) described a point mutation in exon 2 of BMP15, which they associated with prolificacy in Iranian Mehraban and Lori sheep.

In sheep, *BMP15* consists of 2 exons, separated by an intron of approximately 5.4 kb in length. It encodes a prepropeptide of 393 amino acids that contains a predicted amino-terminal signal peptide of 25 amino acids length (Galloway et al., 2000). The signal peptide precedes a 244 amino-acid pro-region and a putative 125 amino-acid carboxy-terminal mature peptide beyond the RRAR protease cleavage site. The ovine BMP15 coding region sequence is 82.9% homologous to that of human and 78.8% homologous to that of mouse. The BMP15 gene is located on the X chromosome, so if a ram carries the gene, all of his daughters will inherit a single copy. This is of benefit in sheep breeding, as for some of the known *BMP15* mutations, heterozygous ewes have been reported to have increased fertility (Galloway et al., 2000; Hanrahan et al., 2004).

In order to ascertain the extent of *BMP15* variation in New Zealand sheep, and whether that variation is associated with litter- size, two regions of the gene were analysed using Polymerase Chain Reaction-Single-Strand Conformation Polymorphism (PCR-SSCP) analysis. Associations with the litter- size were explored statistically in Finnish Landrace sheep, Finnish Landrace x Texel-cross sheep of varying breed proportions, and composite sheep that include a variety of breeds in unknown proportion.

5.2 Materials and Methods

Experimental animals

The data on litter-size data of ewes in 2016 was obtained from one flocks' records. The pedigrees had a maximum depth of five consecutive generations. Sheep without records and whose families could not be identified were omitted. A total of 251 sheep from three different breeds were analysed for this study. All sheep fertility data was derived from this flock which were farmed on pasture and all fed the same way. These included NZ New Zealand Finnish Landrace sheep (n = 148), Finnish Landrace × Texel-cross sheep (n = 45), and composite sheep (of varying breed background; n = 58). Composite sheep are sheep bred from a wide variety of genetic backgrounds based on selection for key production traits. Their background is typically very diverse, and in the case of the sheep described here will include at very least NZ Romney, Texel, East Friesian and Finnish Landrace sheep based on what is known about the history of the flock. The breed proportion is unknown and likely variable from sheep to sheep. These sheep were primarily bred for lamb/meat production and not wool or milk, using the NZ eBV-based system known as Sheep Improvement Limited (SIL).

The blood samples analysed, DNA purification method, PCR amplification, SSCP analysis, genotyping and DNA sequencing were as described in chapter four.

Statistical Analyses

The R programme (Team, 2013) was downloaded from www.r-project.org and used to analyse the data. Analyses included determining the number of lambs born per ewe with different *BMP15* genotypes, and analysis of variant and genotype frequency between the Finnish Landrace, Finnish Landrace × Texel-cross and composite sheep.

Assessment of the effect of the *BMP15* variants on fertility was conducted using an ASREML software V4 (Gilmour et al. 2009) and two models: animal models and sire models. The ASREML software was used to estimate the additive and dominance effects of the nucleotide sequence variation in different models. In the models, the effect of each variant on litter size, relative to the other variant was estimated. For example, to estimate the additive effect of variant *A* relative to the variant B, the genotypes were coded according to the "number of copies" of variant *A*: *AA* = 2; *AB* = 1; and *BB* = 0. For estimating the dominance effect of variant *A*, genotypes were coded according to the "presence" of variant *A: AA* or *AB* = 1; and *BB* = 0.

The best complete models was selected by screening all possible subsets of the following full model:

$$y_{ijkln} = \mu + \alpha_i + Breed_j + AGE_k + G_l + e_{ijkln}$$

$$y_{ijkln} = \mu + S_i + Breed_j + AGE_k + G_l + e_{ijkln}$$

Where y_{ijkln} represents the phenotypic value of litter size of the i^{th} ewe in 2016; μ is the average number of lambs born per ewe; G_l is the additive effect of BMP15 variant, $Breed_j$ is the fixed effect of breed (when the three breeds were analysed together); AGE_k is the ewe's age at birth fitted as covariate; α_i is the random animal effect of ewe i ~N(0, σ2a)) when full pedigree matrix A was fitted in the animal model; Si is the random effect of sire of ewe i ~N(0, σ2s) when the relationships between the sires in Matrix S was fitted in sire model ; and e_{ijkln} is the random residual effect for each observation [~N(0, σ2e)].

5.3 Results

Nucleotide sequencing of homozygous genotypes confirmed that the detected variants for region 1, spanning part of the 5'-UTR, exon 1 and part of intron 1, were two unique DNA sequences. The sequence of *B* revealed a three base pair deletion (CTT) deletion at positions 31 to 33 relative to *A*, leading to a leucine deletion (c.31_33del, p.Leu11del). The deletions were named according to the recommendations of the Human Genome Variation Society recommended nomenclature

(https://varnomen.hgvs.org/), this being noted because the deletion has been recorded previously (Guo et al., 2004, Hanrahan et al., 2004), but named differently as it was prior to the establishment of a unifying nomenclature. It should also be noted that while the deletion is named c.31_33del as recommended by the nomenclature, it could also erroneously be called c.28_30del; p.Leu10del, as the CTT sequence is present in two copies in the non-deletion *A* variant sequence. The variant names chosen in this study *A* and *B*, match with the allele names *A* and *B* used in the Guo et al. (2004) report.). The frequency of variant *A* (without c.31_33del) in Finnish Landrace and Finnish Landrace x Texel-cross and composite sheep observed was 0.77, 0.92, 0.68, respectively while the allelic frequency of variant *B* (c.31_33del) was 0.22, 0.07, 0.31, respectively. Interestingly, the frequency of c.31_33del was very high in the composite sheep. No homozygous *BB* (c.31_33del) Finnish Landrace × Texel-cross sheep were detected. The genotypic frequency of homozygous *BB* sheep (c.31_33del) for the Finnish Landrace and composite sheep was 0.09 and 0.1, respectively. The genotypic frequency of homozygous sheep without c.31_33del and heterozygous sheep was very similar in the composite sheep (0.4). The average litter size for the studied group was 2.47±0.99 in Finnish Landrace, 1.92±0.73 in Finnish Landrace × Texel-cross sheep and 1.93±0.45 in composite sheep (All groups=2.18±0.85).

The association analysis results (Table 7), indicate an association between *BMP15* variation and litter-size including an additive effect and dominance effect (Table 7), but no associations with litter- size were observed for the Finnish Landrace or Finish Landrace x Texel-cross sheep. The estimates for the effect of variant *A* in the composite sheep was -0.26 ± 0.092 (P = 0.008) and -0.22 ± 0.095 (P = 0.026), in the animal and sire models respectively, suggesting homozygous sheep without c.31_33del (variant *A*) had a lower litter- size, while composite sheep with c.31_33del had a higher litter- size. It is apparent that in all models, all the sources of variation included in the models are significant, except the age at birth for the Finnish Landrace x Texel-cross sheep (P > 0.05).

Table 7 suggests suggested that only the presence of c.31_33del (variant *B*) was associated with litter size in the composite sheep (P < 0.001). The presence of the c.31_33del deletion, was associated with an increase in the number of lambs born of 0.44 ± 0.122 (P < 0.001) and 0.40 ± 0.126 (P < 0.003), in the animal and sire models, respectively. All the factors included in these models were highly significant (P < 0.001), and variant *A* was not associated with litter- size when the effect of the *BMP15* variants were fitted as a dominance effect.

Table 7 Estimated effect of *BMP15* variants and nucleotide substitutions fitted as having an additive and dominance effects on number of lambs born per ewe in three groups of NZ sheep

Groups	Models	Type of effect	*BMP15* Variation	P-value	Effect (± se)[a]	Source of variation	
						Group	Ewe age
All Groups	Animal	Additive	*A*	0.806	0.02 ± 0.108	**0.022**	**0.001**
	Animal	Additive	*B*	0.806	-0.02 ± 0.108	**0.027**	**0.001**
	Animal	Dominance	*A*	0.586	0.14 ± 0.272	**0.022**	**0.001**
	Animal	Dominance	*B*	0.97	-0.004 ± 0.1335	**0.027**	**0.001**
	Sire	Additive	*A*	0.811	0.02 ± 0.108	**0.022**	**0.001**
	Sire	Additive	*B*	0.811	-0.02 ± 0.108	**0.027**	**0.001**
	Sire	Dominance	*A*	0.608	0.13 ± 0.272	**0.022**	**0.001**
	Sire	Dominance	*B*	0.968	-0.004 ± 0.1335	**0.027**	**0.001**
Finnish Landrace	Animal	Additive	*A*	0.232	0.24 ± 0.200	-	**0.001**
	Animal	Additive	*B*	0.232	-0.24 ± 0.200	-	**0.001**
	Animal	Dominance	*A*	0.072	0.95 ± 0.520	-	**0.001**
	Animal	Dominance	*B*	0.511	-0.15 ± 0.236	-	**0.001**
	Sire	Additive	*A*	0.333	0.18 ± 0.186	-	**0.001**
	Sire	Additive	*B*	0.333	-0.18 ± 0.186	-	**0.001**
	Sire	Dominance	*A*	0.15	0.76 ± 0.518	-	**0.001**
	Sire	Dominance	*B*	0.586	-0.11 ± 0.221	-	**0.001**
Finnish Landrace x Texel-cross	Animal	Additive	*A*	0.954	-0.02 ± 0.381	-	0.239
	Animal	Additive	*B*	0.954	0.02 ± 0.381	-	0.250
	Animal	Dominance	*A*	0.667	0	-	0.239
	Animal	Dominance	*B*	0.954	0.02 ± 0.381	-	0.250
	Sire	Additive	*A*	0.954	-0.02 ± 0.381	-	0.239
	Sire	Additive	*B*	0.954	0.02 ± 0.381	-	0.250
	Sire	Dominance	*A*	0.67	0	-	0.239
	Sire	Dominance	*B*	0.954	0.02 ± 0.381	-	0.250
Composite sheep	Animal	Additive	*A*	**0.008**	-0.26 ± 0.092	-	**0.002**
	Animal	Additive	*B*	**0.008**	0.26 ± 0.092	-	**0.002**
	Animal	Dominance	*A*	0.689	-0.08 ± 0.216	-	**0.002**
	Animal	Dominance	*B*	**< 0.001**	0.44 ± 0.122	-	**0.002**
	Sire	Additive	*A*	**0.026**	-0.22 ± 0.095	-	**0.002**
	Sire	Additive	*B*	**0.026**	0.22 ± 0.095	-	**0.002**
	Sire	Dominance	*A*	0.749	-0.06 ± 0.204	-	**0.002**
	Sire	Dominance	*B*	**0.003**	0.40 ± 0.126	-	**0.002**

[a]Estimation of the effect +/- standard error of each variant relative to other variants on litter size. $P < 0.05$ in bold type

5.4 Discussion

Various studies have shown that *BMP15* sequence variations, including c.897A>T, c.873C>T, c.1100T>G, c.487_503del, c.950C>T, c.718C>T, c.963G>A, c.1009A>C, c.302_304delCTA, c.301G > T, and c.310insC affect prolificacy in heterozygous ewes and sterility in homozygous ewes (Bodin et al., 2007; Davis, 2005; Demars et al., 2013; Galloway et al., 2000; Hanrahan et al., 2004; Monteagudo et al., 2009). Rams that are carriers of this gene are mated with non-carrier ewes to increase prolificacy and maintain this gene in herds (Davis, 2005).

This study did not find any sequence variation in the sheep studied, other than c.31_33del, which was associated with litter- size in only the composite sheep. The sequence containing the CTT

deletion at nucleotide positions 31-33 of exon 1 of *BMP15* is deposited in GenBank (with the accession number: NC_019484.2), and the deletion was detected in the three different groups of sheep studied.

This deletion in the signal sequence of *BMP15* has been described previously (Galloway et al., 2000; Guo et al., 2004; Hanrahan et al., 2004). It was first reported by Galloway et al. (2000) who suggested that the deletion had no phenotypic effect, an observation that was also made by Hanrahan et al. (2004). In contrast to these studies, Guo et al. (2004) reported the occurrence of c.31_33del in Small Tail Han Sheep, and described how the absence/presence of the extra leucine was associated with *BB* ewes having lower fertility compared to *AA* ewes in their second parity, albeit no effect was observed in the first parity, and neither the *AA* or *BB* ewes were significantly different top the *AB* ewes that had a least squares mean (LSM) value for fertility that fell between the *AA* and *BB* ewes.

This result contrasts with our findings, where the presence of the c.31_33del deletion (*B* variant), was associated with an increase in the number of lambs born of 0.44 ± 0.122 ($P < 0.001$) and 0.40 ± 0.126 ($P < 0.003$), in the animal and sire models, respectively. Taken together, with the observation that c.31_33del did not appear to affect litter- size in the Finnish Landrace and Finnish Landrace x Texel-cross sheep we investigated, then the effect of this deletion in the signal sequence seems to vary from study to study and breed to breed. The low small sample size used in Hanrahan et al. (2004) may however be the reason that no association with fertility was reported, and in the study of Guo et al. 2004 the frequency of the *BB* genotype was 7.5% (18 sheep). It is unclear whether the repeated comparison of genotypes without an apparent correction in this study may have affected the outcome, but with low allele frequencies, the outcomes could be biased by the fertility of individual sheep. In our analysis, 10% of the sheep had the rarer *BB* genotype.

In another study, Yang et al. (2006) described two genotypes (*AA* and *AB*) of *BMP15* in Small Tail Han sheep and Dorset sheep, with the frequencies of *AA* being 0.638 and 0.800, and the frequency of *AB* being 0.362 and 0.200, respectively in the two breeds. Sequencing revealed a CTT deletion at what they described as positions 28-30 of exon 1 of BMP15 gene (i.e. c.31_33del) in genotype *AB* when compared to genotype *AA*. The equated this deletion with the CTT deletion reported previously (Hanrahan et al., 2004), and suggested their preliminarily findings indicated the CTT deletion mutation of BMP15 has had no significant effect on prolificacy for Small Tail Han sheep; this contrasting the findings of Guo et al. (2004).

In humans, Lakhal et al. (2009) have described BMP15 signal sequence variation that leads to the amino acid change p.S5R in a patient with severe ovarian dysfunction, and Rossetti et al. (2009)

described how that change leads to defective production of bioactive protein. This contrasts some of the findings in sheep with the p.Leu11del. Care is needed in making these comparisons though, because not only is the variation in a different part of the signal sequence, but the substitution of serine, which is classified as a polar amino acid with a reactive hydroxyl group, with arginine a charged, aliphatic amino acid; is very likely to have a different effect to the loss of the non-polar aliphatic amino acid leucine in sheep. Additionally, BMP15 also appears to regulate ovulation rate and female fertility in a species-specific manner, being apparently crucial in humans and sheep, and largely trivial in mice where loss-of-function of BMP15 results only in subfertility (Yoshino et al., 2006), with Yan et al. (2001) revealing that BMP15 'knockout' mice are fertile, although fecundity is somewhat reduced. In this respect, Veitia & Caburet (2009) described how while the predicted signal peptide sequences of BMP15 are conserved in mammals, there is also evidence that in some species there has been sequence turnover, but with preservation of functionality, suggesting the accumulation of both neutral and compensatory mutations.

We accept there are several limitations to this study. First, the number of samples of Finnish Landrace (n = 148) and Finnish Landrace x Texel-cross (n = 45) sheep may have been insufficient to detect the influence of *BMP15* c.31_33del on the fertility. However, an effect was detected with the composite sheep (n = 58), albeit analysis of all the sheep together saw the association disappear. This suggests there may be other breed-specific effects that are greater than, and/or override the effect of *BMP15* c.31_33del on fertility. When the three groups of sheep were analysed together, 'group' had a significant effect on the analysis, but unfortunately, given the lack of information about the genetic background of the composite sheep, it would be difficult to conclude anything about specific breed effects, as 'group' is a necessarily encompassing term for the sheep studied. In addition, the lower frequency of the c.31_33del across all three groups would reduce the power to detect an association with litter size, and while known effects were factored into the analyses, environmental factors may also have affected the fertility of the sheep studied, thereby confounding the results.

Use of the identified *BMP15* deletion as a marker to improve reproductive performance in the NZ sheep industry, would appear to be worthy of further study. In effect, these results lay a theoretical foundation to further this type of analysis with more common NZ breeds and crosses, if c.31_33del is present and/or can be introduced from some of the breeds in the sheep studied. If that is done, then effort should also be made to search for other functional variations of BMP15, especially those that do not render sheep infertile when in a homozygous state. The rest of the BMP15 gene, including upstream and downstream nucleotide sequences also needs to be characterised in detail in the common NZ breeds such as the NZ Romney, Perendale and Coopworth.

Chapter 6

Association of bone morphogenetic protein receptor type 1B (*BMPR1B*) variation in two amplified regions with litter-size in New Zealand sheep

6.1 Introduction

Progress in the past two decades in improving sheep reproduction has been achieved through the discovery of functional variation in fertility genes. One of the best known genes or genetic effects is 'Booroola', which is now known to be a specific sequence change (c.746A>G, p.Arg249Glu) in the Bone morphogenetic protein receptor type 1B (BMPR1B) gene (*BMPR1B*, Ensembl: ENSOART00000018678.1, also known as *Alk6*, *SKR6*, *ALK-6*, *AMDD*, *BDA2*, *BDA1D*, *CDw293*, *Acvrlk6*, *BMPR-1B*, *BMPR-IB*, *CFK-43a*, *AI385617* and *AV355320*).

Initially focus was placed on the Booroola phenotype (Piper & Bindon, 1987), where the presence of the 'Booroola gene' not only increased ovulation rate by nearly three standard deviations per copy, but also increases litter-size. Davis et al. (1982) found that ewes carrying one copy of the Booroola gene from their parent produced 1.5 more ovules and one more lamb, than ewes lacking this gene. In 2001, three research groups from AgResearch (New Zealand), Institut National de la Recherche Agronomique (INRA) (France), and Edinburgh (United Kingdom) discovered that ewes carrying the Booroola gene had sequence variation in the gene for BMPR1B (Davis et al.,2005).

Chu et al. (2007) found evidence that *BMPR1B* c.746A>G (p.Arg249Glu) in both heterozygous and homozygous sheep increased ovulation rate and litter-size, whereas in wild-type sheep no effect was observed on ovulation rate and litter-size. A study conducted by Bodin et al. (2007) found that Lacaune sheep in France lacked mutations in the BMPR1B gene, while a study of nine breeds of sheep (Guan et al., 2006) found that only the Hu and Chinese Merino sheep breeds carried *BMPR1B* variation that was associated with litter-size. Interestingly, only genotype BB (Booroola) was found in the Hu breed, but all three genotypes including ++, +B and BB were detected in Merino sheep. In another study on eight prolific sheep breeds, Davis et al. (2002) reported that only Garole Indian and Javanese sheep carried the c.746A>G mutation. Heaton et al. (2017) identified two new sequence variations c.360G>A and c.1180A>C that enhanced fertility and prolificacy in Katahdin and Romanov sheep. Abdoli et al. (2018) reported prolificacy in sheep was not affected by a new synonymous mutation (g.66496G>A) in exon 8 in Iranian Fat-Tailed Sheep.

Little is known about the role of *BMPR1B* variations in controlling fertility in NZ sheep breeds. Therefore, this investigation aimed to characterise variation in *BMPR1B* in NZ breeds and ascertain whether it affected litter size.

6.2 Material and methods

Ethics statement

This research project was carried out following the requirements of the Animal Welfare Act 1999 (NZ Government) for research involving animals.

Blood samples and DNA purification

Three hundred and thirty-five ewes from three populations were genotyped for the sequence variations in two fragments of *BMPR1B*. The samples were collected from one flock in Canterbury from three New Zealand sheep breeds including Finnish Landrace (n = 165), Finnish Landrace × Texel-cross (n = 56), composite sheep (of varying breed background based on NZ Romney-type genetics n = 114) to analyse associations between polymorphisms in BMPR1B gene and litter-size in NZ sheep breeds.

FTA cards (Whatman BioScience, Middlesex, UK) were used for blood collection from a small incision in the ear of the sheep. DNA was extracted from the blood samples by punching a 1.2 mm disc from the FTA card, followed by genomic DNA purification using a two-step procedure described by (Zhou et al., 2006). To begin this process, the FTA card punch was placed in tubes containing 200 µL of 20 mM NaOH, left for 20 to 30 minutes at 62 °C, or until the disk became white. All the liquid was then removed and the disk equilibrated in 200 µL of 1× TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). After this, the liquid was again removed, and the disks were left to air dry in the tubes overnight.

PCR amplification and PCR-Single Strand Conformation Polymorphism (PCR-SSCP) analysis

The Polymerase Chain Reaction (PCR) primers used in this study were as follows: Forward: 5'-CAACGAGGATGGGTATTAGTCG-3' and Reverse: 5'- TCAGATCTCGATGGGCAATTG-3' designed to amplify a 394 bp fragment of *BMPR1B* exon 9 and intron 8; and Forward 5'-GATCGAACCCGAGTCTCTTG-3'and Reverse: 5'-AGCTGGCCTCCTCTGTAGTG-3' designed to amplify a 338 bp fragment of exon 8 and part of intron 7. The primers were designed manually based on GenBank sequence NC_019463.2 to amplify fragments that were reported to contain sequence variation in other studies.

The PCR amplifications were performed in a 15-µL reaction containing the genomic DNA on one 1.2-mm punch of FTA card, 0.25 µM of each primer, 150 µM of each dNTP (Bioline, London, UK), 0.3 mM Mg2+, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1× reaction buffer supplied with the enzyme. Amplification was undertaken as follows: initial denaturation at 94 °C for 2 min, followed by 35 cycles 94 °C for 30 s (denaturation), 60 °C for 30 s (annealing), 72 °C for 30 s (elongation), with a final extension step at 72 °C for 5 min.

For SSCP analysis, a 0.7 µL aliquot of each amplicon was mixed with 7 µL of loading dye (98% Formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol), and after denaturation at 95 oC for 5 minutes, the samples were rapidly cooled on wet ice and immediately loaded on to 16 cm × 18 cm, 12% acrylamide: bisacrylamide (37.5:1; Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad), at 350 V for 18 hours at 7 oC in 0.5x TBE buffer. The DNA fragments were visualized using a silver nitrate staining method (Byun et al. 2009). Briefly, the gels were bathed in a solution of 10% ethanol, 0.5% acetic acid and 0.2% AgNO3 for 10 minutes. Next, the gels were rinsed with distilled water then developed with a solution of 3% NaOH and 0.1% HCOH until dark-staining bands appeared on the yellow background of the gel.

Genotyping and sequencing

PCR amplicons from two samples representing different banding patterns from sheep that appeared to be homozygous were sequenced in both directions at the Lincoln University DNA Sequencing Facility, NZ to confirm that variants detected represented unique sequences. Variants that were only found in heterozygous sheep were sequenced using an approach described by (Gong et al., 2011). A band corresponding to each variant was excised as a gel slice from the polyacrylamide gel, macerated, and then used as a template for re-amplification with the original primers. This second amplicon was then sequenced. Sequence alignments, translations, and phylogenetic analysis were carried out using DNAMAN (version 5.2.10, Lynnon BioSoft, Vaudreuil, Canada).

Statistical analysis

The genotype analysis was performed in R programming software (Team, 2013) to examine the number of lambs born per ewe with different BMPR1B genotypes, including an analysis of variant and genotype in the pure Finnish Landrace and Finnish Landrace × Texel-cross ewes and composite sheep.

Assessment of the presence or absence of each of the *BMPR1B* variants on fertility was conducted using an ASREML approach (Gilmour et al. 2009) and using two models: animal models and sire models. The ASREML software was used to estimate the additive and dominance effects of single

SNPs in different models. In the models, the effect of each variant on litter-size, relative to the other variants was estimated. For example to estimate the additive effect of variant *A* relative to the other variants (*B* and *C*), the genotypes were coded according to the "number of copies" of variant *A*: *AA* = 2; *AB* or *AC* = 1; and *BB*, *BC* or *CC* = 0. For estimating the dominance effect of variant *A*, genotypes were coded according to the "presence" of variant *A*: *AA* or *AB* or *AC* = 1; and *BB* or *BC* or *CC* = 0.

The best complete models were selected by screening all possible subsets of the following full model:

1*)* $y_{ijkln} = \mu + \alpha_i + Breed_j + AGE_k + G_l + e_{ijkln}$

2) $y_{ijkln} = \mu + S_i + Breed_j + AGE_k + G_l + e_{ijkln}$

Where y_{ijkln} represents the phenotypic value of litter-size of the i^{th} ewe in 2016; μ is the average number of lambs born per ewe; G_l is the additive and dominance effect of *BMPR1B* variant, $Breed_j$ is the fixed effect of breed (when the three breeds were analysed together); AGE_k is the ewe's age at birth fitted as covariate; α_i is the random animal effect of ewe i when full pedigree matrix A was fitted in the animal model~N (0, σ^2_a); S_i is the random effect of sire ewe i ~N(0, σ2s) when the relationships between the sires in Matrix S was fitted in sire model ; and e_{ijkln} is the random residual effect for each observation [~N(0, σ2e)].

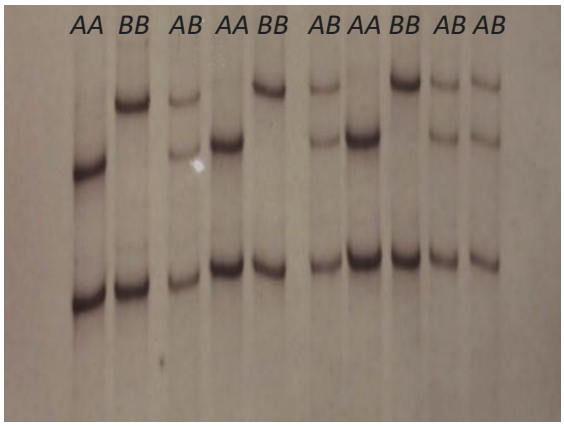
6.3 Results

A 394 bp fragment spanning the partial exon 9 and intron 8 and a 338 bp of exon 8 and intron 7 regions of BMPR1B in 335 sheep, belonging to three NZ breeds was amplified (Figure 11). The PCR-SSCP analysis and nucleotide sequencing revealed two banding patterns (*A*, *B*), and three combinations of these banding patterns (*AA*, *AB*, *BB*) in the intron-8/exon 9 amplicon from BMPR1B, and three banding patterns (*A*, *B*, and *C*) and six combinations (*AA*, *AB*, *AC*, *BB*, *BC* and *CC*) were found for the intron 7/exon 8 amplicons.

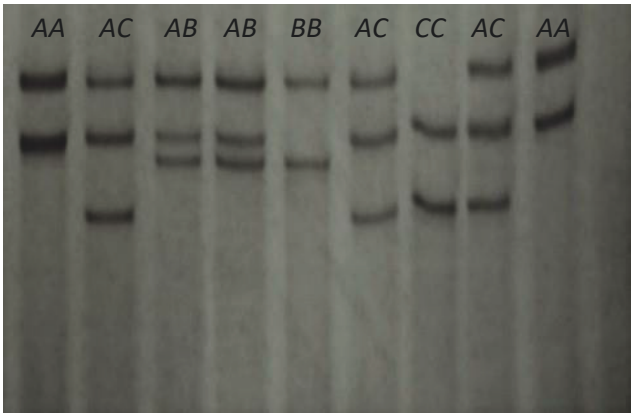
Sequencing of the amplicons identified five unique DNA sequences. The sequencing results revealed sequence variation c.1032T>C (rs159952533) in the exon 9/intron 8 amplicon, and c.754-144G>A, c.754-88G>A, c.762G>A, c.754-31C>T and c.765G>A in the exon 8/intron 7 amplicon. The T>C substitution at position 1032, would be silent and not change the corresponding amino acid (p.Tyr344). In exon 8; c.754-144G>A, c.754-88G>A and c.754-31C>T are non-coding sequence variations, while c.762G>A and c.765G>A are synonymous substitutions (p.Arg254) and (p.Thr255) respectively.

This investigation revealed that all of the sequence variations –c.1032T>C in the studied fragment of exon 9/intron 8, and c.754-144G>A, c.754-88G>A, c.762G>A, c.754-88G>A, c.754-31C>T and c.765G>A in the amplified region of exon 8/intron 7 were observed in all the investigated groups,

with the exception that variant *B* and *C* of exon 8/intron 7 was not detected in Finnish Landrace x Texel-cross, and all sheep were homozygous (*AA*) for this region.



(a)



(b)

SNP	*Variant* *A*	*B*
c.1032T>C	T	C

(c)

SNP	*Variant* *A*	*B*	*C*
c.754-144G>A	A	G	G
c.754-88G>A	G	A	G
c.762G>A	G	A	G
c.754-88G>A	G	G	A
c.754-31C>T	C	C	T
c.765G>A	G	G	A

(d)

Figure 11 The gel patterns for PCR-SSCP analysis of a 394 bp fragment of intron-8/exon 9 of *BMPR1B*. Two banding patterns representing two variants (*A* and *B*) were identified in both homozygous and heterozygous forms. b) Sequence analysis revealed one sequence variation. c) PCR-SSCP patterns for a 338 bp fragment of exon 8/intron7 of *BMPR1B*. Three banding patterns representing three variants (*A*, *B* and *C*) were identified in both homozygous and heterozygous forms indicating homozygous variants A (well 1), B (well 5), and C (well 7). d) Sequence analysis revealed 6 sequence variations in the exon8/ intron7 of *BMPR1B*.

Table 8 The observed variant and genotype frequencies for the variants of an exon nine and exon eight fragments of Bone Morphogenetic Protein Receptor type 1B (*BMPR1B*) in Finnish Landrace, Finnish Landrace x Texel-cross, composite NZ sheep

Group	Genotype Frequencies			Allele Frequencies		Genotype Frequencies						Allele Frequencies		
	exon 9/intron 8-9					exon 8/intron 7-8								
	AA	*AB*	*BB*	*A*	*B*	*AA*	*AB*	*AC*	*BB*	*BC*	*CC*	*A*	*B*	*C*
Finnish Landrace	0.68	0.26	0.048	0.82	0.18	0.72	0.10	0.08	0.04	0.04	0.02	0.81	0.11	0.07
Finnish Landrace x Texel-cross	0.625	0.33	0.035	0.79	0.20	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
Composite sheep	0.88	0.10	0.008	0.93	0.061	0.62	0.21	0.09	0.03	0.05	0.00	0.77	0.16	0.07

From Table 8, we can see allelic and genotypic frequency of *BMPR1B* variants in the Finnish Landrace and Finnish Landrace x Texel-cross sheep and the composite sheep. Variant *A* in both exons was the most common one in all the groups. The genotype *CC* for exon 8/intron 7 was absent in the Finnish Landrace x Texel-cross and composite sheep. The variant *A* was the most common, and the *AA* genotype was the most common in all the studied groups of sheep. It is also noted that variant *B* in exon 8/intron 7-8 was more common than *C*; consequently the *AA* genotype was the most prevalent followed by the *AB* then the *AC* genotypes. Overall, 7% of the Finnish Landrace and composite sheep were carriers of variant *C*, which was detected at a very low frequency in these two groups. The average litter size for the studied group was 2.43±1.04 in Finnish Landrace; 1.83±0.66 in Finnish Landrace × Texel-cross sheep; and 1.83±0.49 in the composite ewes (All groups=2.14±0.87).

Association studies assessing the effect of the detected variants on litter-size were carried out for the 335 sheep. The estimated impact of *BMPR1B* variants (intron-8/exon 9) fitted as having an additive effect on the number of lambs born per ewe in three groups of New Zealand sheep are summarised in (

Table 9). What stands out is that although the estimated p-values obtained for both variants with in each breed were similar, neither of the sequence variants in this region had a significant effect on litter-size in Finnish Landrace × Texel-cross sheep (P > 0.05). The estimate for the impact of variant *B* was 0.04 ± 0.186 (p = 0.82) and -0.03 ± 0.169 (P = 0.80) in both Finnish Landrace x Texel-cross and Finnish Landrace respectively. The effect of variant B was 0.01 ± 0.106 when all groups were analysed together, i.e., the breed effect was included in the model

Table 9 Estimated effect of *BMPR1B* variants and nucleotide substitutions in exon 8/intron7 fitted as having an additive and dominance effects on number of lambs born per ewe in three groups of New Zealand sheep

	groups	Type of effect	*BMPR1B* variation	P-value	Effect (±se)[a]	Source of variation	
						Group	Ewe age
All Groups	Animal	Additive	A	0.549	-0.06 ± 0.116	**0.039**	**.001**
	Animal	Additive	B	0.747	-0.04 ± 0.146	**0.037**	**.001**
	Animal	Additive	C	0.215	0.21 ± 0.170	**0.038**	**.001**
	Animal	Dominance	A	0.47	-0.18 ± 0.260	**0.038**	**.001**
	Animal	Dominance	B	0.596	-0.09 ± 0.172	**0.036**	**.001**
	Animal	Dominance	C	0.15	0.28 ± 0.197	**0.039**	**.001**
	Sire	Additive	A	0.425	-0.08 ± 0.111	**.001**	**.001**
	Sire	Additive	B	0.9	-0.01 ± 0.142	**.001**	**.001**
	Sire	Additive	C	0.182	0.22 ± 0.165	**.001**	**.001**
	Sire	Dominance	A	0.424	-0.21± 0.262	**.001**	**.001**
	Sire	Dominance	B	0.747	-0.05 ± 0.165	**.001**	**.001**
	Sire	Dominance	C	0.116	0.30 ± 0.191	**.001**	**.001**
Finnish Landrace	Animal	Additive	A	0.726	-0.06 ± 0.195	-	**.001**
	Animal	Additive	B	0.861	-0.04 ± 0.239	-	**.001**
	Animal	Additive	C	0.504	0.17 ± 0.266	-	**.001**
	Animal	Dominance	A	0.972	-0.01 ± 0.455	-	**.001**
	Animal	Dominance	B	0.888	-0.04 ± 0.309	-	**.001**
	Animal	Dominance	C	0.434	0.28 ± 0.361	-	**.001**
	Sire	Additive	A	0.53	-0.11 ± 0.175	-	**.001**
	Sire	Additive	B	0.945	0.01 ± 0.225	-	**.001**
	Sire	Additive	C	0.392	0.22 ± 0.258	-	**.001**
	Sire	Dominance	A	0.973	0.01 ± 0.447	-	**.001**
	Sire	Dominance	B	0.859	0.05 ± 0.282	-	**.001**
	Sire	Dominance	C	0.282	0.37 ± 0.350	-	**.001**
Composite sheep	Animal	Additive	A	0.855	-0.01 ± 0.104	-	**0.071**
	Animal	Additive	B	0.398	-0.11 ± 0.138	-	**0.103**
	Animal	Additive	C	0.162	0.23 ± 0.167	-	**0.042**
	Animal	Dominance	A	0.399	-0.17 ± 0.210	-	**0.058**
	Animal	Dominance	B	0.301	-0.16 ± 0.152	-	0.131
	Animal	Dominance	C	0.162	0.23 ± 0.167	-	**0.042**
	Sire	Additive	A	0.688	-0.04 ± 0.101	-	**0.019**
	Sire	Additive	B	0.651	-0.05 ± 0.131	-	**0.021**
	Sire	Additive	C	0.211	0.20 ± 0.162	-	**0.020**
	Sire	Dominance	A	0.36	-0.19 ± 0.213	-	**0.018**
	Sire	Dominance	B	0.484	-0.10 ± 0.145	-	**0.020**
	Sire	Dominance	C	0.211	0.20 ± 0.162	-	**0.020**

aEstimation of the effect +/- standard error of each variant relative to other variants on litter size. P < 0.05 in bold type.

The estimated effect of identified variants in intron 7/exon 8 and nucleotide substitutions fitted as having an additive effect are summarised in Table 10.

Table 10 Estimated effect of *BMPR1B* variants and nucleotide substitutions in (intron 8/exon 9) fitted as having an additive and dominance effects on number of lambs born per ewe in three groups of New Zealand sheep

Groups	Models	Type of effect	*BMPR1B* variation	P-value	Effect (± se)[a]	Source of variations Group	ewe age
All Groups	Animal	Additive	A	0.85	-0.01 ± 0.106	**0.021**	**.001**
	Animal	Additive	B	0.85	0.01 ± 0.106	**0.021**	**.001**
	Animal	Dominance	A	0.715	0.09 ± 0.261	**0.018**	**.001**
	Animal	Dominance	B	0.661	0.05 ± 0.136	**0.019**	**.001**
	Sire	Additive	A	0.603	-0.05 ± 0.104	**0.001**	**.001**
	Sire	Additive	B	0.603	0.05 ± 0.104	**0.001**	**.001**
	Sire	Dominance	A	0.787	0.06 ± 0.266	**0.001**	**.001**
	Sire	Dominance	B	0.432	0.10 ± 0.133	**0.001**	**.001**
Finnish Landrace	Animal	Additive	A	0.809	0.03 ± 0.169	-	**.001**
	Animal	Additive	B	0.809	-0.03 ± 0.169	-	**.001**
	Animal	Dominance	A	0.674	0.16 ± 0.393	-	**.001**
	Animal	Dominance	B	0.942	-0.01 ± 0.225	-	**.001**
	Sire	Additive	A	0.706	-0.06 ± 0.165	-	**.001**
	Sire	Additive	B	0.706	0.06 ± 0.165	-	**.001**
	Sire	Dominance	A	0.888	0.05 ± 0.397	-	**.001**
	Sire	Dominance	B	0.567	0.12 ± 0.219	-	**.001**
Finnish Landrace x Texel-cross	Animal	Additive	A	0.822	-0.04 ± 0.186	-	0.404
	Animal	Additive	B	0.822	0.04 ± 0.186	-	0.404
	Animal	Dominance	A	0.74	-0.16 ± 0.499	-	0.404
	Animal	Dominance	B	0.902	0.02 ± 0.227	-	0.405
	Sire	Additive	A	0.82	-0.04 ± 0.186	-	0.406
	Sire	Additive	B	0.82	0.04 ± 0.186	-	0.406
	Sire	Dominance	A	0.739	-0.16 ± 0.499	-	0.404
	Sire	Dominance	B	0.9	0.02 ± 0.227	-	0.404
Composite sheep	Animal	Additive	A	0.81	-0.04 ± 0.188	-	**0.039**
	Animal	Additive	B	0.81	0.04 ± 0.188	-	**0.039**
	Animal	Dominance	A	0.982	0.01 ± 0.496	-	**0.039**
	Animal	Dominance	B	0.741	0.08 ± 0.249	-	**0.039**
	Sire	Additive	A	0.752	-0.05 ± 0.183	-	**0.014**
	Sire	Additive	B	0.752	0.05 ± 0.183	-	**0.014**
	Sire	Dominance	A	0.996	-0.002 ± 0.4838	-	**0.015**
	Sire	Dominance	B	0.677	0.10 ± 0.242	-	**0.014**

[a]Estimation of the effect +/- standard error of each variant relative to other variants on litter size. $P < 0.05$ in bold type.

No significant association was found between the number of lambs born and detected sequence variations in exon 8/intron 7 across all the studied groups. It is apparent from Table 10 that both fixed effects are significant in different models, except the age at lambing in the model for composite

sheep (P > 0.05). Interestingly, all of the genotyped ewes were homozygous for Finnish Landrace × Texel-cross sheep and no variation was detected in this amplified region for this breed. The only modest (but not statistically significant, p ≈ 0.162) association was the effect of variant *C* on increased litter-size in composite sheep (0.23 ± 0.167). The impact of variant *B* in Finnish Landrace -0.04 ± 0.239 (P = 0.861) is more or less identical to the effect of this variant when all groups were analysed together -0.04 ± 0.146 (P =0.747).

6.4 Discussion

Due to the low heritability of sheep reproductive traits, many studies have been conducted using scans of candidate fertility genes in different sheep breeds. As discussed in previous chapters, the identification of nucleotide sequence variation in candidate genes (*GDF9*, *BMP15* and *BMPR1B*) has been revealed to play a crucial role in phenotypic variation in fertility. Among the detected variations in the BMPR1B gene, the variant c.746A>G, was first found in Booroola Merino sheep (Mulsant et al., 2001). This mutation is not only reported in Booroola Merino sheep, but also in Garole sheep (Davis et al., 2002), Javanese sheep (Davis et al., 2002), Iranian Kalehkoohi sheep (Mahdavi et al., 2014), and small-tailed Han (Chu et al., 2007). The BMPR1B Booroola mutation has an additive effect on ovulation rates and a dominant effect for litter-size.

The results of the current study indicate that *BMPR1B* is a polymorphic gene in NZ sheep breeds. In the sheep investigated, *BMPR1B* was variable, with six single nucleotide polymorphisms detected. These were c.1032T>C (rs159952533) in the exon 9/intron 8 region, and c.754-144G>A (rs411048486), c.754-88G>A (rs399052946), c.762G>A(rs408447622), c.754-31C>T(rs421837112) and c.765G>A (rs427897187) detected in the exon 8/intron 7 region in Finnish Landrace, Finnish Landrace x Texel-cross sheep and composite sheep, when compared to the GenBank reference sequences (NC_019484.2) .

The exon 8/intron 7 of the *BMPR1B* was found to be monomorphic in Finnish Landrace x Texel-cross; therefore, no association between the detected variant and litter-size could be established in this breed. Although variation in the gene was detected in the other groups of sheep, we did not find any evidence of association between the variations in the two fragments of *BMPR1B* and litter-size across all groups. Moreover, when analysis was conducted across all groups, using two different animal and sire models, again no sequence variation or haplotype was associated with litter-size in the New Zealand sheep that were studied. The absence of a significant association between variation in *BMPR1B* and litter-size in the three New Zealand sheep breeds/types, was possibly because the number of available records for numbers of lambs born per ewe for our genotyped samples was too low to reach statistical significance. Also the low frequency of some detected variants meant that, although they may have been present, they were not discernible in the studied sample.

Although three of the nucleotide variations (c.754-144G>A, c.754-88G>A and c.754-31C>T) identified in this study was in the non-coding region and thus does not usually result in expression of the gene, the effect of these sequence variations on litter-size may exist through a link with another susceptible gene or induction of aberrant splicing of mRNA resulting in mutant mRNA production (Shen et al., 2001).

Abdoli et al. (2013) analysed the data for the association between sequence variations detected in exon 8 of *BMPR1B* and litter-size and concluded that sequence variations (c.66496G>A) detected in Iranian Mehraban sheep in exon 8 were significantly associated with litter-size, but in this study when the exon 8/intron 7 region was studied, no associations were found.

Our analysis revealed that the two sequence variations identified in exon 8 (c.762G>A and c.765G>A), very close to the position of the c.746A>G variant (Booroola), would be silent and thus potentially less likely to affect fertility in NZ sheep breeds. They might however be in linkage disequilibrium with another causal mutation in *BMPR1B*. Moreover, linkage disequilibrium of the identified sequence variations in this study and c.746A>G and other mutations in other loci should be considered and investigated. It should be noted that analysing the combined effect of multiple genes or loci on litter-size is very important in complex quantitative traits like reproductive traits (An et al., 2013), hence the association between multiple loci in different genes also needs to be considered and analysed.

Notwithstanding the lack of significant association between detected variants and litter-size in this work, future studies could investigate the importance of variations in other fragments of this gene and other candidate gene like growth differentiation factor 9 (*GDF9*). Additionally, the failure to identify associations in the present study could suggest that larger sample sizes are needed to make the statistical analyses more robust. To help confirm or refute the current findings, further prospective understanding of the association of variations with litter-size in these two regions of BMPR1B in different sheep breeds should also be considered.

In conclusion, our results suggest that *BMPR1B* is polymorphic in some New Zealand sheep breeds, but the genetic variations in this gene were not associated with litter-size. These results are informative and representative of an essential step in directing future research for detecting variations in other major genes and their association with litter-size. The results also demonstrate that the PCR-SSCP approach can efficiently identify variations in all domestic animals, including different sheep breeds and also help to direct future research on relationships between fertility and BMPR1B variation.

Chapter 7

General discussion, conclusions and future directions

Increased meat production in New Zealand can be obtained by increasing litter-size using both traditional phenotype-based breeding and functional variations in key genes to enable for genotype-assisted selection. It needs to be considered that a high number of lambs born, can result in lower birth weights and increased post-natal mortality, hence an optimum number of lambs born is desirable for different New Zealand sheep production systems. Such decisions by farmers will be dictated by costs associated with incorporation of such functional variations in marker-assisted selection programmes.

This thesis began with the aim of identifying functional variations in candidate genes for increased litter-size in New Zealand sheep breeds, and thus to provide tools for selection in sheep breeding programs. The genes studied were chosen because they had been shown to affect litter-size or number of lambs born per ewe in previous studies with different breeds. The study utilized multiple models to establish the association between genetic variation and litter size, and has reported a range of results supporting three candidate genes for a role in improving litter size in some of New Zealand sheep, namely *bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) genes.* . The three breeds were chosen for association study derived from a single large ewe flock of the three breeds (farmed on pasture and all fed the same way) so there is no flock effect to correct for (albeit we have corrected for breed). The phenotypic records were available only for these three groups and some records were omitted due to lack of sire or dam information, and because no records of litter size for most sheep in all years were available we only include the data of litter size for 2016. In this study, Although SSCP-sequencing is a suitable detection method for eukaryotic gene regions, there are some limitations in that it can only detect sequence variation in fragments under 400 bp in size, but if the DNA fragments of interest are less than 400 bp in length, then SSCP generally offers suitable discriminatory ability and reproducibility. A MassArray sequencing technique may allow more cost-effective and faster genotyping of multiple SNPs (spread across the entire gene region for multiple genes) in one go. In this thesis, in the models to find the association between the detected variants and litter size, the random polygenic effects were calculated by performing BLUP (Best Linear Unbiased Prediction) analyses. ASREML software was used to solve the mixed model equations and the additive and dominance effects of each variant on litter size was estimated using two models: animal models and sire models. In the sire models, the polygenic effects were calculated using the sires' pedigree information and it is assumed that all mated dams are of similar genetic merit. In the animal models, polygenic effects are calculated using full pedigree information that can eliminate the issues in sire model. However, the main advantage of sire model

is reduction in the number of equations that need to be solved, compared to the animal models, since only sire information is used to calculate the polygenic effects (i.e. breeding values). Further, if the sire's information is recorded more accurately, the polygenic predictions in sire models tend to be less affected by errors in pedigree.

The first gene investigated in this study was *GDF9* with some variations identified that had the potential to contribute to the functionality with respect to improving litter size in Finnish Landrace X Texel crosses. The findings of chapter two provided a better understanding of genetic variation in *GDF9* in NZ sheep, and may ultimately be of value in controlling reproductive performance in sheep. Chapter two discussed the consequences of variation in the GDF9 gene, and how the most significant benefit of the functional variations may be realised, both for commercial breeders and the sheep themselves. Sequence analyses of the three variants detected in *GDF9* exon 2 fragment revealed three sequence variations: c.978A>G, c.994G>A and c.1111G>A. Analysis of litter size data for Finnish Landrace × Texel- cross-bred sheep revealed an association between litter-size and the sequence variation c.1111G>A, but this was not observed for the Finnish Landrace sheep and the composite sheep. When all the sheep were analysed together, the presence of c.1111A was associated with increased litter-size compared to ewes that had c.1111G. Litter-size did not differ between sheep with and without c.994A in all three breeds investigated breeds.

Validation of this apparent association in the Finnish Landrace x Texel-cross sheep by crossing this breed with other breeds without this mutation would be beneficial. Certainly for *GDF9*, the functional sequence variation (c.1111A) present in our studied samples would appear to be useful in selecting for improvement in the number of lambs born per ewe because the direction of effect on litter size was in line with the previous studies. Further study is needed to better understand the effect of the mutations associated with changes in protein structure on the number of lambs born per ewe.

The other gene studied was *BMP15*. The c.31-33del in exon 1 is widely distributed in New Zealand sheep breeds, including White Dorper, Finnish Landrace sheep, the Finnish Landrace × Texel-cross sheep, Perendale, Merino, Romney, Corriedale, Wiltshire, Dohne, Coopworth, Easycare, Southdown, Shropshire, Lleyn and composite sheep. The fact that the c.58-60del deletion appears in the coding region highlights the necessity for the effect on the resulting protein to be investigated; hence the additive and dominance effect of variants on litter-size were estimated using both animal and sire models for composite sheep. This identified an association between litter-size and the c.31_33del in composite sheep. Analysing all groups together, the litter-size did not differ significantly between sheep breeds regardless of the presence of c.31_33del. The results suggest that the c.31_33del sequence variation could possibly be a genetic marker for improving fecundity in New Zealand sheep,

but more work will be needed. No relationship was found between c.31_33del and litter-size in Finnish Landrace or Finnish Landrace x Texel-cross sheep.

The chapter six explored genetic variations within the *BMPR1B*. The sequencing results revealed six sequence variations including c.1032T>C in the studied fragment of exon 9 and intron 8, and c.754-144G>A, c.754-88G>A, c.762G>A,, c.754-31C>T and c.765G>A in the amplified region of exon 8 and intron 7. While variations in *BMPR1B* have been confirmed in different sheep breeds, no association was found between the detected variations and litter-size. The number of sheep studied may have been a limiting factor to obtaining statistical significance, as samples with phenotypic data were limited to Finnish Landrace, Finnish Landrace x Texel-cross and composite sheep.

Overall, the sample size will need to be increased for all these candidate genes to ascertain their importance in reproduction and fertility in New Zealand sheep. The presence of some polymorphisms detected in the investigated genes (*GDF9* and *BMP15*) in this study could possibly be used in marker-assisted selection (MAS) to improve fertility in New Zealand sheep breeds, but the research would benefit significantly from further investigation and with more sheep.

The result of this thesis have occurred at the same time as the development of a commercial gene-marker within the GDF9 gene to improve litter size. A commercial gene-market for GDF9 gene, which has been successful marketed to farmers across Australian and New Zealand to improve sheep fertility now available at Gene marker laboratory at Lincoln University.

The frequency of c.1111A was very high in the composites on farms 2, 3 and 4. Validation of the effect of this allele could be done using large number of sheep from those farms and also further studies on other genes that may influence fecundity in various New Zealand sheep breeds should be carried out.

Appendix A

GDF9 gene

A.1 Sequence alignment of submitted sequence and GenBank sequence (AF078545.2) of exon two of *GDF9*

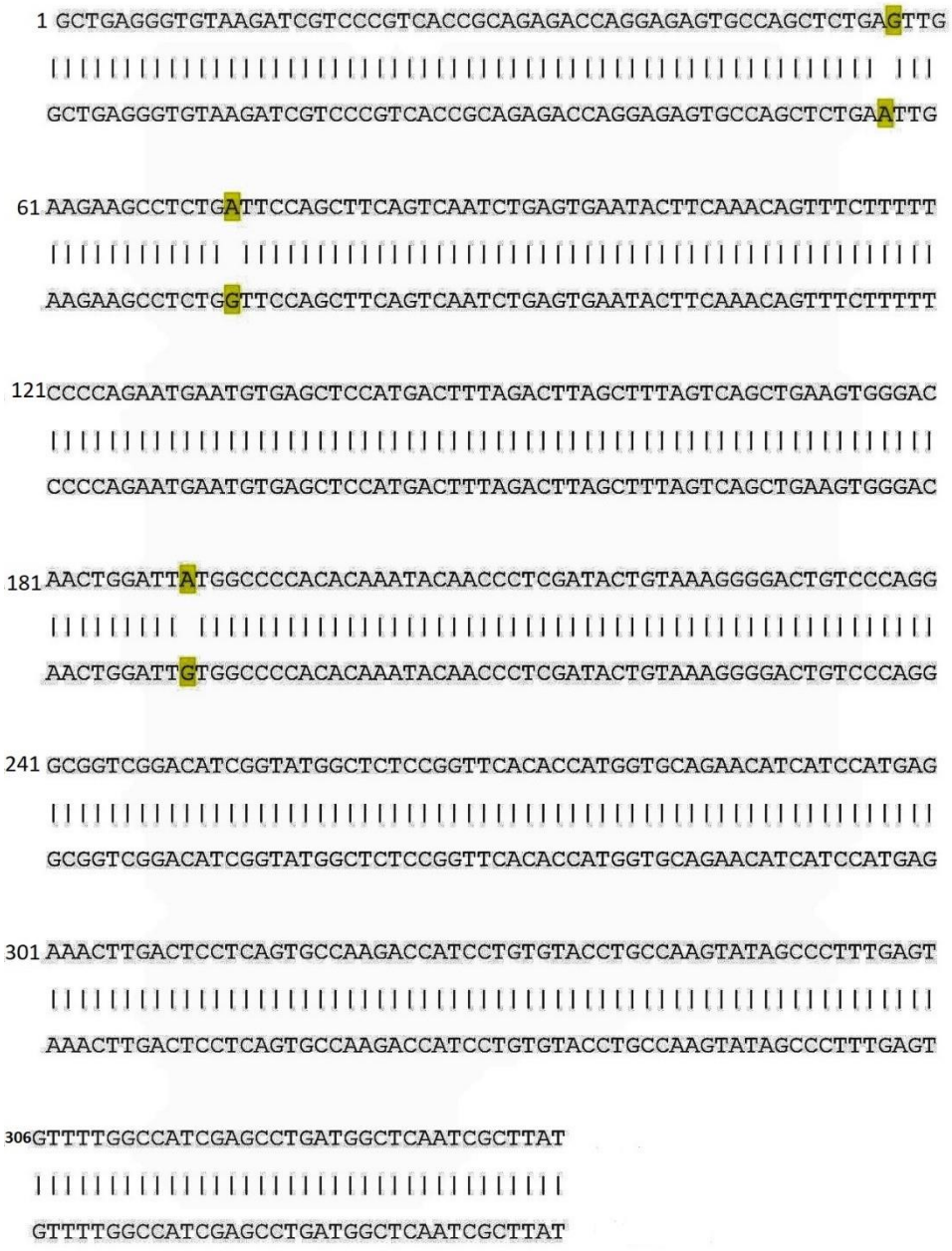


Table A. 1 The genotypic and phenotypic information for three different groups in for *GDF9* exon 2

ID	Sire	Dam	Group	Genotype	NLB in 2016
285	249	250	Finnish Landrace	AA	2
289	242	159	Finnish Landrace	AA	5
314	259	250	Finnish Landrace	AA	3
319	288	289	Finnish Landrace	AA	3
323	286	295	Finnish Landrace	AA	2
326	286	297	Finnish Landrace	AA	2
332	259	204	Finnish Landrace	AA	4
336	312	251	Finnish Landrace	AA	1
338	288	257	Finnish Landrace	AA	3
339	317	315	Finnish Landrace	AA	2
341	288	284	Finnish Landrace	AA	4
343	317	318	Finnish Landrace	AA	2
344	317	319	Finnish Landrace	AA	2
350	286	324	Finnish Landrace	AA	4
366	352	158	Finnish Landrace	AA	3
375	282	355	Finnish Landrace	AA	4
380	282	364	Finnish Landrace	AA	4
384	363	266	Finnish Landrace	AA	2
385	282	366	Finnish Landrace	AA	3
390	363	255	Finnish Landrace	AB	4
395	363	298	Finnish Landrace	AC	2
399	363	290	Finnish Landrace	AC	3
401	363	287	Finnish Landrace	AA	5
403	363	307	Finnish Landrace	AA	4
407	311	354	Finnish Landrace	AC	3
409	376	375	Finnish Landrace	AA	2
411	311	360	Finnish Landrace	AA	2
414	363	289	Finnish Landrace	AA	3
417	378	377	Finnish Landrace	AA	2
418	378	379	Finnish Landrace	AA	2
419	312	380	Finnish Landrace	AA	1
422	381	314	Finnish Landrace	AA	2
424	381	316	Finnish Landrace	AA	3
425	282	382	Finnish Landrace	AA	3
427	378	384	Finnish Landrace	AC	2
428	378	379	Finnish Landrace	AA	3
445	363	266	Finnish Landrace	AA	1
447	363	306	Finnish Landrace	AA	3
458	363	275	Finnish Landrace	AA	3

472	286	462	Finnish Landrace	AA	4
477	464	253	Finnish Landrace	AA	1
479	464	293	Finnish Landrace	AA	3
481	378	467	Finnish Landrace	AA	2
482	378	468	Finnish Landrace	AC	2
483	378	469	Finnish Landrace	AC	4
484	464	315	Finnish Landrace	AA	3
485	378	470	Finnish Landrace	AA	1
486	464	253	Finnish Landrace	AA	1
487	464	382	Finnish Landrace	AC	2
488	312	471	Finnish Landrace	AA	1
489	312	472	Finnish Landrace	AA	1
490	464	293	Finnish Landrace	AA	4
494	464	360	Finnish Landrace	AA	1
506	282	355	Finnish Landrace	AA	4
512	464	401	Finnish Landrace	AA	3
513	378	476	Finnish Landrace	AA	2
514	311	366	Finnish Landrace	AC	4
524	376	477	Finnish Landrace	AA	2
526	376	402	Finnish Landrace	AA	2
527	312	333	Finnish Landrace	AA	2
528	376	478	Finnish Landrace	AA	2
529	312	313	Finnish Landrace	AA	3
530	317	128	Finnish Landrace	AA	2
531	378	403	Finnish Landrace	AA	3
569	412	390	Finnish Landrace	BC	2
570	413	477	Finnish Landrace	AA	2
581	413	416	Finnish Landrace	AA	1
589	412	481	Finnish Landrace	AC	2
593	408	472	Finnish Landrace	AA	1
597	410	419	Finnish Landrace	AA	1
612	408	337	Finnish Landrace	AA	2
614	312	423	Finnish Landrace	AA	2
617	412	485	Finnish Landrace	AC	2
633	413	380	Finnish Landrace	AA	2
645	410	342	Finnish Landrace	AA	1
646	410	488	Finnish Landrace	AC	2
647	410	488	Finnish Landrace	AA	1
235	219	124	Finnish Landrace X Texel cross	AA	1
325	294	296	Finnish Landrace X Texel cross	AC	2
345	294	293	Finnish Landrace X Texel cross	AA	2
346	294	320	Finnish Landrace X Texel cross	AA	2
347	219	321	Finnish Landrace X Texel cross	AC	2
348	219	320	Finnish Landrace X Texel cross	AA	2
349	219	323	Finnish Landrace X Texel cross	AA	2
351	219	326	Finnish Landrace X Texel cross	AA	1
370	356	258	Finnish Landrace X Texel cross	AA	3
389	356	158	Finnish Landrace X Texel cross	AA	2

392	356	158	Finnish Landrace X Texel cross	AA	2
431	357	386	Finnish Landrace X Texel cross	AA	3
434	219	387	Finnish Landrace X Texel cross	AC	3
435	219	388	Finnish Landrace X Texel cross	AC	2
437	357	386	Finnish Landrace X Texel cross	AA	1
438	294	389	Finnish Landrace X Texel cross	AA	2
439	294	355	Finnish Landrace X Texel cross	AA	2
440	357	390	Finnish Landrace X Texel cross	AB	1
446	294	370	Finnish Landrace X Texel cross	AA	2
448	219	392	Finnish Landrace X Texel cross	AC	3
450	394	322	Finnish Landrace X Texel cross	AA	2
452	394	325	Finnish Landrace X Texel cross	AA	1
453	327	395	Finnish Landrace X Texel cross	AC	3
455	394	397	Finnish Landrace X Texel cross	AA	2
456	219	398	Finnish Landrace X Texel cross	AA	2
457	219	393	Finnish Landrace X Texel cross	AA	2
473	357	463	Finnish Landrace X Texel cross	AA	3
491	219	462	Finnish Landrace X Texel cross	AA	2
492	294	473	Finnish Landrace X Texel cross	AA	2
493	219	474	Finnish Landrace X Texel cross	AA	1
495	394	475	Finnish Landrace X Texel cross	AA	2
558	219	479	Finnish Landrace X Texel cross	AC	3
561	219	392	Finnish Landrace X Texel cross	AC	0
658	219	345	Finnish Landrace X Texel cross	AA	1
660	219	325	Finnish Landrace X Texel cross	CC	2
662	219	392	Finnish Landrace X Texel cross	AC	2
678	433	434	Finnish Landrace X Texel cross	AC	2
681	219	307	Finnish Landrace X Texel cross	AA	1
685	219	354	Finnish Landrace X Texel cross	AA	2
689	219	389	Finnish Landrace X Texel cross	AA	2
693	219	438	Finnish Landrace X Texel cross	AC	2
703	219	475	Finnish Landrace X Texel cross	AA	1
704	219	365	Finnish Landrace X Texel cross	AC	2
712	433	348	Finnish Landrace X Texel cross	AC	2
748	433	453	Finnish Landrace X Texel cross	AC	1
753	433	457	Finnish Landrace X Texel cross	AC	1
782	219	475	Finnish Landrace X Texel cross	AA	1
198	139	138	composite sheep	AB	2
212	54	198	composite sheep	AB	2
223	115	200	composite sheep	AA	2
229	54	198	composite sheep	AA	2
234	123	218	composite sheep	BB	2
334	113	308	composite sheep	AA	2
373	115	361	composite sheep	AA	2
374	54	362	composite sheep	AA	2
406	113	374	composite sheep	AA	2
497	54	220	composite sheep	BC	3
502	113	222	composite sheep	AB	2

508	113	126	composite sheep	AA	2
510	305	224	composite sheep	AA	3
511	113	198	composite sheep	AB	2
515	305	301	composite sheep	AB	2
516	310	330	composite sheep	AA	2
517	305	212	composite sheep	AA	2
518	305	198	composite sheep	AB	2
519	310	223	composite sheep	AA	2
520	305	374	composite sheep	AA	2
522	305	127	composite sheep	AA	2
532	129	226	composite sheep	AA	2
534	305	228	composite sheep	AB	3
535	305	130	composite sheep	AB	2
536	227	229	composite sheep	AA	2
537	305	230	composite sheep	AA	2
538	305	404	composite sheep	AA	2
539	305	231	composite sheep	AA	2
540	305	231	composite sheep	AA	2
541	305	131	composite sheep	AA	2
542	305	232	composite sheep	AA	2
543	227	233	composite sheep	AB	1
544	305	215	composite sheep	AB	2
545	227	223	composite sheep	AA	2
546	227	331	composite sheep	AA	2
547	227	121	composite sheep	AA	1
548	227	373	composite sheep	AA	2
550	305	405	composite sheep	AA	2
551	305	406	composite sheep	AA	2
552	305	374	composite sheep	AA	1
553	305	334	composite sheep	AA	2
554	305	334	composite sheep	AA	2
555	305	127	composite sheep	AA	1
556	305	127	composite sheep	AA	1
557	394	335	composite sheep	AB	2
766	0	215	composite sheep	BB	1
767	0	130	composite sheep	BB	1
768	227	198	composite sheep	AA	2
771	227	515	composite sheep	AA	2
772	227	518	composite sheep	AB	1
774	0	545	composite sheep	AA	2
775	0	131	composite sheep	AB	1
776	0	230	composite sheep	AC	1
777	227	497	composite sheep	AC	0
778	0	542	composite sheep	AA	1
779	0	553	composite sheep	AA	2
780	0	550	composite sheep	AA	1
781	0	555	composite sheep	AA	1

Appendix B

BMP15 gene

B.1 Sequence of amplified *BMP15* fragment from exon 1 (NC_019484.2)

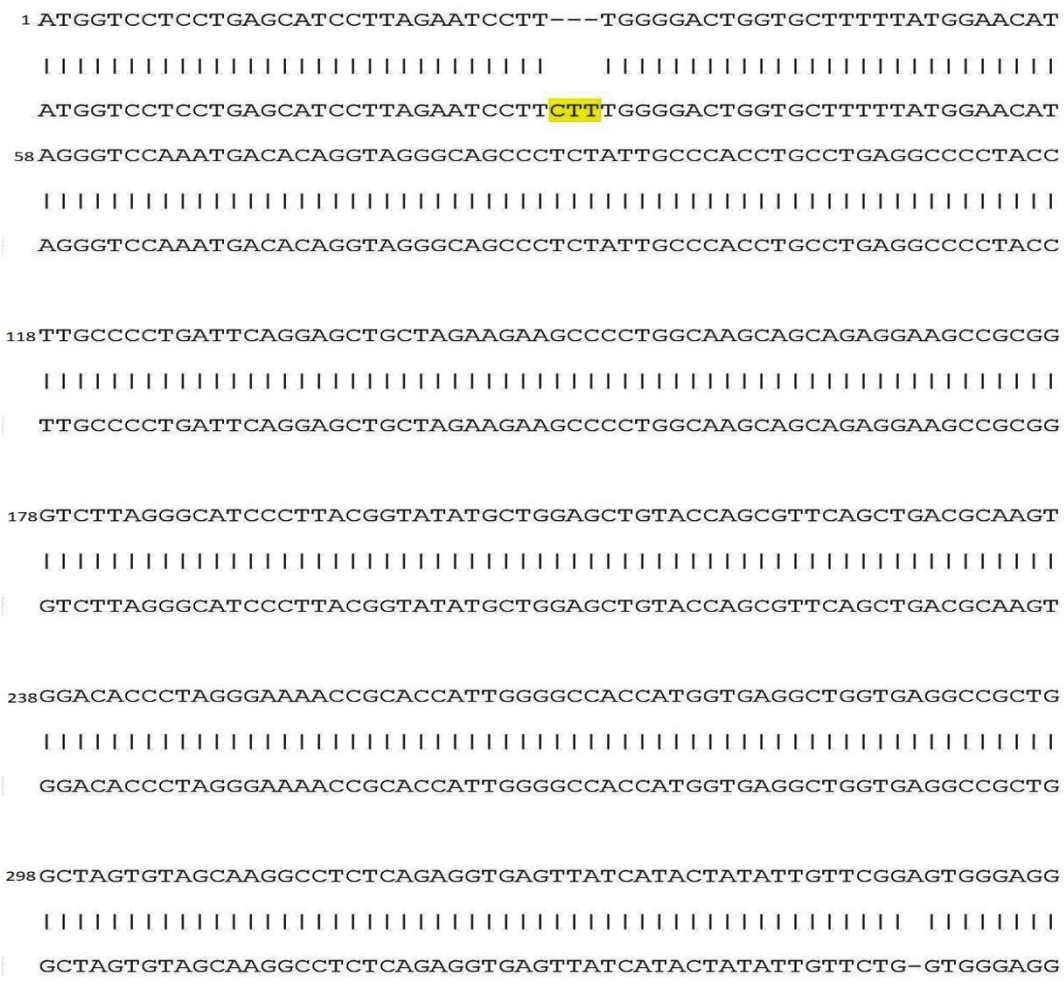


Table B. 2 The genotypic and phenotypic information for three different groups in *BMP15* (exon 1)

ID	Sire	Dam	Group	Genotype	NLB in 2016
285	249	250	Finnish Landrace	AA	2
314	259	250	Finnish Landrace	AA	3
319	288	289	Finnish Landrace	AA	3
323	286	295	Finnish Landrace	AB	2
326	286	297	Finnish Landrace	AB	2
332	259	204	Finnish Landrace	AA	4
336	312	251	Finnish Landrace	AB	1
338	288	257	Finnish Landrace	AA	3
339	317	315	Finnish Landrace	AA	2
341	288	284	Finnish Landrace	AA	4
342	312	257	Finnish Landrace	AA	2
343	317	318	Finnish Landrace	AB	2
344	317	319	Finnish Landrace	AA	2
350	286	324	Finnish Landrace	AB	4
366	352	158	Finnish Landrace	AA	3
375	282	355	Finnish Landrace	AB	4
380	282	364	Finnish Landrace	AA	4
384	363	266	Finnish Landrace	AA	2
385	282	366	Finnish Landrace	AA	3
390	363	255	Finnish Landrace	AA	4
395	363	298	Finnish Landrace	AA	2
401	363	287	Finnish Landrace	AB	5
403	363	307	Finnish Landrace	AA	4
407	311	354	Finnish Landrace	AA	3
409	376	375	Finnish Landrace	BB	2
411	311	360	Finnish Landrace	AA	2
414	363	289	Finnish Landrace	AB	3
417	378	377	Finnish Landrace	AB	2
418	378	379	Finnish Landrace	AB	2
422	381	314	Finnish Landrace	AA	2
424	381	316	Finnish Landrace	AA	3
425	282	382	Finnish Landrace	AA	3
427	378	384	Finnish Landrace	AB	2
428	378	379	Finnish Landrace	AB	3
429	376	385	Finnish Landrace	AB	1
445	363	266	Finnish Landrace	AA	1
447	363	306	Finnish Landrace	AA	3
458	363	275	Finnish Landrace	AA	3
471	286	461	Finnish Landrace	AB	3
472	286	462	Finnish Landrace	AA	4
477	464	253	Finnish Landrace	AB	1
479	464	293	Finnish Landrace	AA	3

481	378	467	Finnish Landrace	AB	2
482	378	468	Finnish Landrace	BB	2
483	378	469	Finnish Landrace	AB	4
484	464	315	Finnish Landrace	AA	3
485	378	470	Finnish Landrace	BB	1
486	464	253	Finnish Landrace	AB	1
487	464	382	Finnish Landrace	AA	2
488	312	471	Finnish Landrace	AA	1
490	464	293	Finnish Landrace	AA	4
494	464	360	Finnish Landrace	AA	1
506	282	355	Finnish Landrace	AA	4
512	464	401	Finnish Landrace	AA	3
513	378	476	Finnish Landrace	AA	2
514	311	366	Finnish Landrace	AA	4
524	376	477	Finnish Landrace	AB	2
526	376	402	Finnish Landrace	AB	2
527	312	333	Finnish Landrace	AB	2
528	376	478	Finnish Landrace	AB	2
529	312	313	Finnish Landrace	AA	3
530	317	128	Finnish Landrace	AA	2
531	378	403	Finnish Landrace	AB	3
569	412	390	Finnish Landrace	AA	2
570	413	477	Finnish Landrace	AB	2
581	413	416	Finnish Landrace	AA	1
589	412	481	Finnish Landrace	AB	2
593	408	472	Finnish Landrace	AB	1
597	410	419	Finnish Landrace	AA	1
612	408	337	Finnish Landrace	AA	2
614	312	423	Finnish Landrace	AA	2
617	412	485	Finnish Landrace	AB	2
633	413	380	Finnish Landrace	AA	2
235	219	124	Finnish Landrace X Texel cross	AA	1
325	294	296	Finnish Landrace X Texel cross	AA	2
346	294	320	Finnish Landrace X Texel cross	AA	2
347	219	321	Finnish Landrace X Texel cross	AB	2
348	219	320	Finnish Landrace X Texel cross	AA	2
351	219	326	Finnish Landrace X Texel cross	AA	1
370	356	258	Finnish Landrace X Texel cross	AA	3
389	356	158	Finnish Landrace X Texel cross	AA	2
392	356	158	Finnish Landrace X Texel cross	AA	2
431	357	386	Finnish Landrace X Texel cross	AA	3
434	219	387	Finnish Landrace X Texel cross	AA	3
435	219	388	Finnish Landrace X Texel cross	AA	2
437	357	386	Finnish Landrace X Texel cross	AB	1
438	294	389	Finnish Landrace X Texel cross	AA	2
439	294	355	Finnish Landrace X Texel cross	AB	2
440	357	390	Finnish Landrace X Texel cross	AA	1
441	294	387	Finnish Landrace X Texel cross	AA	2

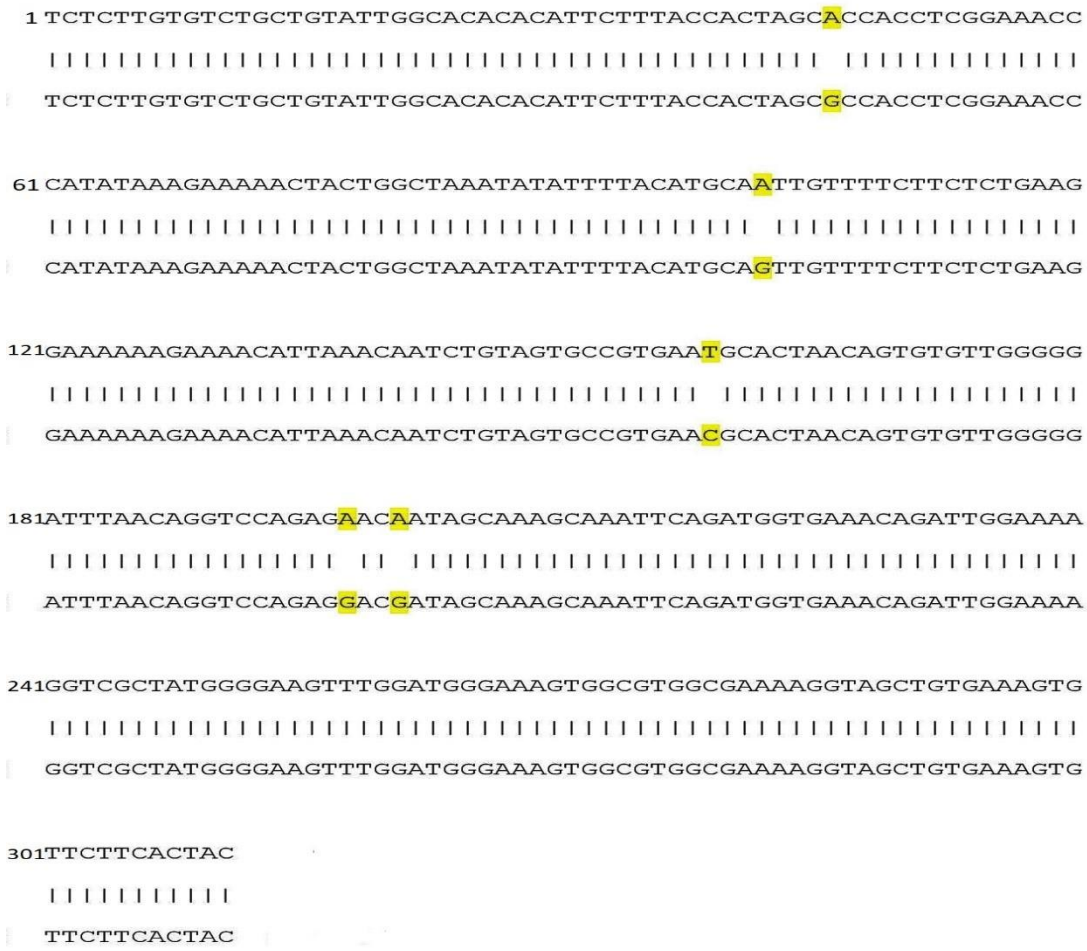
444	294	391	Finnish Landrace X Texel cross	AA	2
446	294	370	Finnish Landrace X Texel cross	AA	2
448	219	392	Finnish Landrace X Texel cross	AA	3
449	219	393	Finnish Landrace X Texel cross	AA	2
450	394	322	Finnish Landrace X Texel cross	AA	2
452	394	325	Finnish Landrace X Texel cross	AA	1
453	327	395	Finnish Landrace X Texel cross	AA	3
454	219	396	Finnish Landrace X Texel cross	AA	1
455	394	397	Finnish Landrace X Texel cross	AA	2
456	219	398	Finnish Landrace X Texel cross	AA	2
457	219	393	Finnish Landrace X Texel cross	AA	2
473	357	463	Finnish Landrace X Texel cross	AB	3
475	294	465	Finnish Landrace X Texel cross	AA	1
491	219	462	Finnish Landrace X Texel cross	AA	2
492	294	473	Finnish Landrace X Texel cross	AB	2
493	219	474	Finnish Landrace X Texel cross	AA	1
495	394	475	Finnish Landrace X Texel cross	AA	2
558	219	479	Finnish Landrace X Texel cross	AA	3
561	219	392	Finnish Landrace X Texel cross	AA	0
223	115	200	composite sheep	AA	2
234	123	218	composite sheep	AB	2
331	305	120	composite sheep	AB	3
334	113	308	composite sheep	AA	2
373	115	361	composite sheep	AB	2
374	54	362	composite sheep	AA	2
406	113	374	composite sheep	AA	2
498	123	198	composite sheep	BB	2
499	115	125	composite sheep	AA	2
502	113	222	composite sheep	AB	2
508	113	126	composite sheep	AA	2
509	113	223	composite sheep	AA	1
510	305	224	composite sheep	AB	3
511	113	198	composite sheep	AB	2
515	305	301	composite sheep	AA	2
517	305	212	composite sheep	BB	2
518	305	198	composite sheep	AB	2
519	310	223	composite sheep	AA	2
520	305	374	composite sheep	AB	2
522	305	127	composite sheep	AB	2
532	129	226	composite sheep	AA	2
533	227	330	composite sheep	AA	2
534	305	228	composite sheep	AB	3
536	227	229	composite sheep	AA	2
537	305	230	composite sheep	BB	2
538	305	404	composite sheep	BB	2
539	305	231	composite sheep	BB	2
540	305	231	composite sheep	AA	2
541	305	131	composite sheep	AB	2

542	305	232	composite sheep	AB	2
543	227	233	composite sheep	AA	1
544	305	215	composite sheep	BB	2
545	227	223	composite sheep	AA	2
546	227	331	composite sheep	AB	2
547	227	121	composite sheep	AA	1
548	227	373	composite sheep	AB	2
550	305	405	composite sheep	AB	2
551	305	406	composite sheep	AB	2
552	305	374	composite sheep	AA	1
553	305	334	composite sheep	AB	2
554	305	334	composite sheep	AB	2
555	305	127	composite sheep	AA	1
556	305	127	composite sheep	AA	1
557	394	335	composite sheep	AA	2

Appendix C

BMPR1B gene

C.1 Sequences of amplified *BMPR1B* fragments from exon 8/intron7 (NC_019463.2)



C.2 Sequences of amplified *BMPR1B* fragments from exons exon 9/intron8 (NC_019463.2)

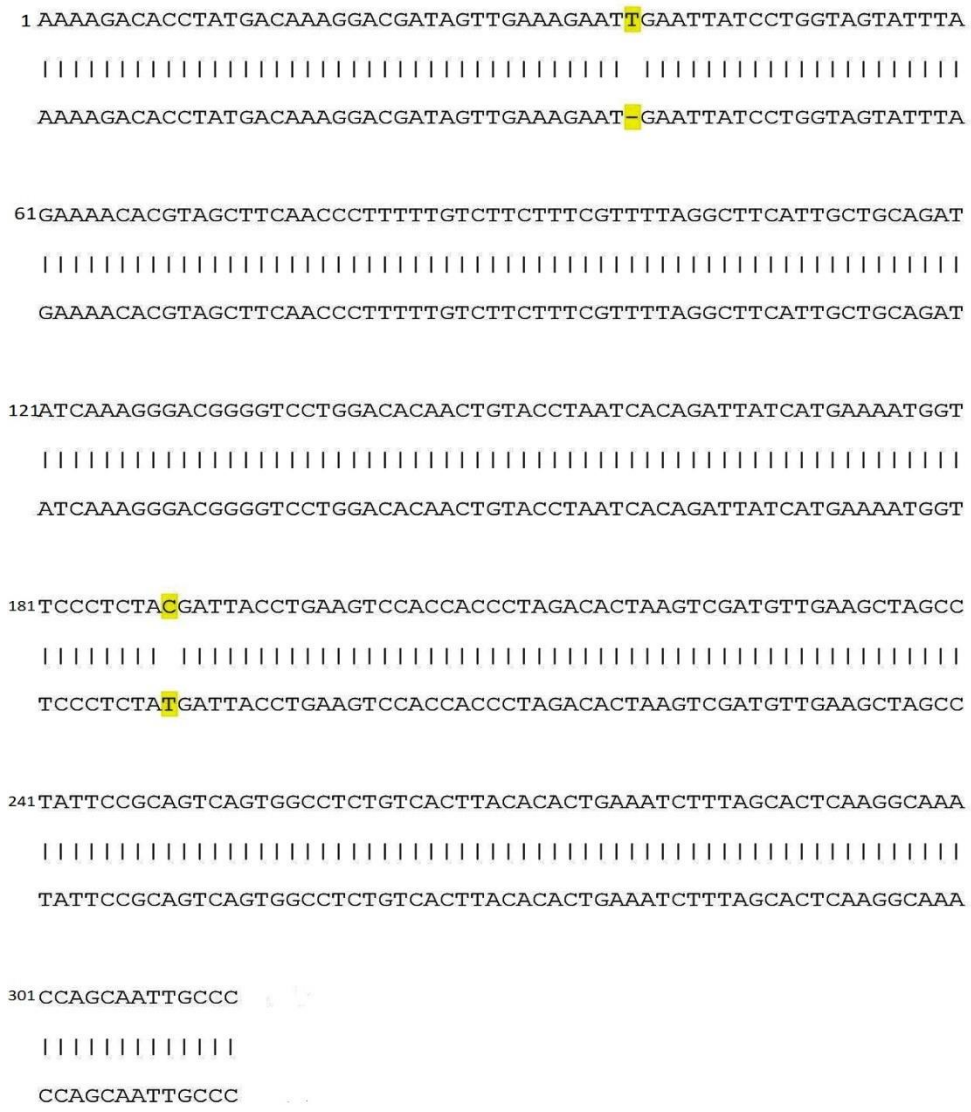


Table C. 3 The genotypic and phenotypic information for three different groups in BMPR1B gene exon 8/intron7

ID	Sire	Dam	Group	Genotype	NLB in 2016
285	249	250	Finnish Landrace	AA	2
289	242	159	Finnish Landrace	AA	5
314	259	250	Finnish Landrace	AA	3
319	288	289	Finnish Landrace	AA	3
323	286	295	Finnish Landrace	AA	2
326	286	297	Finnish Landrace	AA	2
332	259	204	Finnish Landrace	AA	4
336	312	251	Finnish Landrace	AA	1
338	288	257	Finnish Landrace	AA	3
339	317	315	Finnish Landrace	AA	2
341	288	284	Finnish Landrace	AA	4
342	312	257	Finnish Landrace	AA	2
343	317	318	Finnish Landrace	AA	2
350	286	324	Finnish Landrace	AA	4
366	352	158	Finnish Landrace	AC	3
375	282	355	Finnish Landrace	AA	4
380	282	364	Finnish Landrace	AA	4
384	363	266	Finnish Landrace	AC	2
385	282	366	Finnish Landrace	BB	3
390	363	255	Finnish Landrace	AA	4
395	363	298	Finnish Landrace	AA	2
399	363	290	Finnish Landrace	AC	3
401	363	287	Finnish Landrace	AA	5
403	363	307	Finnish Landrace	AC	4
407	311	354	Finnish Landrace	AA	3
409	376	375	Finnish Landrace	AA	2
411	311	360	Finnish Landrace	AA	2
414	363	289	Finnish Landrace	AA	3
417	378	377	Finnish Landrace	AB	2
418	378	379	Finnish Landrace	AC	2
419	312	380	Finnish Landrace	AA	1
422	381	314	Finnish Landrace	AB	2
424	381	316	Finnish Landrace	AA	3
425	282	382	Finnish Landrace	BB	3
427	378	384	Finnish Landrace	CC	2
428	378	379	Finnish Landrace	AB	3
429	376	385	Finnish Landrace	AA	1
445	363	266	Finnish Landrace	AA	1

447	363	306	Finnish Landrace	AA	3
458	363	275	Finnish Landrace	CC	3
471	286	461	Finnish Landrace	AA	3
472	286	462	Finnish Landrace	AA	4
477	464	253	Finnish Landrace	AA	1
479	464	293	Finnish Landrace	AA	3
481	378	467	Finnish Landrace	AB	2
482	378	468	Finnish Landrace	AB	2
483	378	469	Finnish Landrace	AB	4
484	464	315	Finnish Landrace	AA	3
485	378	470	Finnish Landrace	AB	1
486	464	253	Finnish Landrace	AA	1
487	464	382	Finnish Landrace	AA	2
488	312	471	Finnish Landrace	AA	1
489	312	472	Finnish Landrace	AA	1
490	464	293	Finnish Landrace	AA	4
494	464	360	Finnish Landrace	AA	1
506	282	355	Finnish Landrace	AA	4
512	464	401	Finnish Landrace	AA	3
513	378	476	Finnish Landrace	AA	2
514	311	366	Finnish Landrace	AC	4
524	376	477	Finnish Landrace	AA	2
526	376	402	Finnish Landrace	AA	2
527	312	333	Finnish Landrace	AA	2
528	376	478	Finnish Landrace	AA	2
529	312	313	Finnish Landrace	AA	3
530	317	128	Finnish Landrace	AA	2
531	378	403	Finnish Landrace	AB	3
569	412	390	Finnish Landrace	AA	2
570	413	477	Finnish Landrace	AB	2
581	413	416	Finnish Landrace	AB	1
589	412	481	Finnish Landrace	AA	2
593	408	472	Finnish Landrace	AA	1
597	410	419	Finnish Landrace	AA	1
612	408	337	Finnish Landrace	AA	2
614	312	423	Finnish Landrace	AA	2
617	412	485	Finnish Landrace	AA	2
633	413	380	Finnish Landrace	AA	2
645	410	342	Finnish Landrace	AA	1
646	410	488	Finnish Landrace	AA	2
647	410	488	Finnish Landrace	AA	1
235	219	124	Finnish Landrace X Texel cross	AA	1
325	294	296	Finnish Landrace X Texel cross	AA	2
345	294	293	Finnish Landrace X Texel cross	AA	2
346	294	320	Finnish Landrace X Texel cross	AA	2
347	219	321	Finnish Landrace X Texel cross	AA	2
348	219	320	Finnish Landrace X Texel cross	AA	2
351	219	326	Finnish Landrace X Texel cross	AA	1

389	356	158	Finnish Landrace X Texel cross	AA	2
392	356	158	Finnish Landrace X Texel cross	AA	2
431	357	386	Finnish Landrace X Texel cross	AA	3
434	219	387	Finnish Landrace X Texel cross	AA	3
435	219	388	Finnish Landrace X Texel cross	AA	2
437	357	386	Finnish Landrace X Texel cross	AA	1
438	294	389	Finnish Landrace X Texel cross	AA	2
439	294	355	Finnish Landrace X Texel cross	AA	2
441	294	387	Finnish Landrace X Texel cross	AA	2
444	294	391	Finnish Landrace X Texel cross	AA	2
446	294	370	Finnish Landrace X Texel cross	AA	2
448	219	392	Finnish Landrace X Texel cross	AA	3
450	394	322	Finnish Landrace X Texel cross	AA	2
452	394	325	Finnish Landrace X Texel cross	AA	1
453	327	395	Finnish Landrace X Texel cross	AA	3
454	219	396	Finnish Landrace X Texel cross	AA	1
455	394	397	Finnish Landrace X Texel cross	AA	2
456	219	398	Finnish Landrace X Texel cross	AA	2
457	219	393	Finnish Landrace X Texel cross	AA	2
473	357	463	Finnish Landrace X Texel cross	AA	3
475	294	465	Finnish Landrace X Texel cross	AA	1
491	219	462	Finnish Landrace X Texel cross	AA	2
492	294	473	Finnish Landrace X Texel cross	AA	2
493	219	474	Finnish Landrace X Texel cross	AA	1
495	394	475	Finnish Landrace X Texel cross	AA	2
558	219	479	Finnish Landrace X Texel cross	AA	3
561	219	392	Finnish Landrace X Texel cross	AA	0
449	219	393	Finnish Landrace X Texel cross	AA	2
198	139	138	composite sheep	AA	2
212	54	198	composite sheep	AA	2
223	115	200	composite sheep	AB	2
229	54	198	composite sheep	AA	2
234	123	218	composite sheep	AA	2
334	113	308	composite sheep	AB	2
373	115	361	composite sheep	AA	2
374	54	362	composite sheep	AA	2
406	113	374	composite sheep	AB	2
497	54	220	composite sheep	AA	3
498	123	198	composite sheep	AA	2
499	115	125	composite sheep	AA	2
502	113	222	composite sheep	AB	2
510	305	224	composite sheep	AC	3
511	113	198	composite sheep	AB	2
515	305	301	composite sheep	AC	2
516	310	330	composite sheep	AA	2
517	305	212	composite sheep	AC	2
518	305	198	composite sheep	AC	2
519	310	223	composite sheep	AB	2

520	305	374	composite sheep	AC	2
522	305	127	composite sheep	AA	2
532	129	226	composite sheep	AA	2
533	227	330	composite sheep	AA	2
534	305	228	composite sheep	AB	3
535	305	130	composite sheep	AA	2
537	305	230	composite sheep	AC	2
538	305	404	composite sheep	AA	2
539	305	231	composite sheep	AA	2
540	305	231	composite sheep	AC	2
541	305	131	composite sheep	BC	2
542	305	232	composite sheep	AB	2
543	227	233	composite sheep	AB	1
544	305	215	composite sheep	AA	2
545	227	223	composite sheep	BB	2
546	227	331	composite sheep	AA	2
547	227	121	composite sheep	AB	1
548	227	373	composite sheep	AA	2
550	305	405	composite sheep	BC	2
551	305	406	composite sheep	BC	2
552	305	374	composite sheep	AB	1
553	305	334	composite sheep	BC	2
554	305	334	composite sheep	AA	2
555	305	127	composite sheep	AA	1
556	305	127	composite sheep	AA	1
557	394	335	composite sheep	AA	2
766	0	215	composite sheep	AA	1
768	227	198	composite sheep	AA	2
769	0	406	composite sheep	AA	1
770	0	406	composite sheep	AA	1
771	227	515	composite sheep	AA	2
772	227	518	composite sheep	AA	1
773	0	758	composite sheep	AA	2
774	0	545	composite sheep	AA	2
775	0	131	composite sheep	AA	1
776	0	230	composite sheep	AA	1

Table C. 2 The genotypic and phenotypic information for three different groups in BMPR1B gene (exon9/intron8)

ID	Sire	Dam	Group	Genotype	NLB in 2016
285	249	250	Finnish Landrace	AA	2
289	242	159	Finnish Landrace	AA	5
314	259	250	Finnish Landrace	AA	3
319	288	289	Finnish Landrace	AA	3
323	286	295	Finnish Landrace	AA	2
326	286	297	Finnish Landrace	AA	2
332	259	204	Finnish Landrace	AB	4
336	312	251	Finnish Landrace	AA	1
338	288	257	Finnish Landrace	AA	3
339	317	315	Finnish Landrace	AA	2
341	288	284	Finnish Landrace	AA	4
342	312	257	Finnish Landrace	AA	2
343	317	318	Finnish Landrace	AA	2
344	317	319	Finnish Landrace	BB	2
350	286	324	Finnish Landrace	AA	4
366	352	158	Finnish Landrace	AB	3
375	282	355	Finnish Landrace	AA	4
380	282	364	Finnish Landrace	AA	4
384	363	266	Finnish Landrace	AB	2
385	282	366	Finnish Landrace	BB	3
390	363	255	Finnish Landrace	AB	4
395	363	298	Finnish Landrace	AB	2
399	363	290	Finnish Landrace	AA	3
401	363	287	Finnish Landrace	AB	5
403	363	307	Finnish Landrace	AA	4
407	311	354	Finnish Landrace	AA	3
409	376	375	Finnish Landrace	AA	2
411	311	360	Finnish Landrace	AA	2
414	363	289	Finnish Landrace	AB	3
417	378	377	Finnish Landrace	AA	2
418	378	379	Finnish Landrace	AB	2
419	312	380	Finnish Landrace	AA	1
422	381	314	Finnish Landrace	AA	2
424	381	316	Finnish Landrace	AB	3
425	282	382	Finnish Landrace	AA	3
427	378	384	Finnish Landrace	AA	2
428	378	379	Finnish Landrace	AB	3
429	376	385	Finnish Landrace	AB	1
445	363	266	Finnish Landrace	BB	1
447	363	306	Finnish Landrace	AA	3
458	363	275	Finnish Landrace	AA	3
471	286	461	Finnish Landrace	AA	3
472	286	462	Finnish Landrace	AA	4
477	464	253	Finnish Landrace	AA	1

479	464	293	Finnish Landrace	AA	3
481	378	467	Finnish Landrace	AB	2
482	378	468	Finnish Landrace	AA	2
483	378	469	Finnish Landrace	AA	4
484	464	315	Finnish Landrace	AA	3
485	378	470	Finnish Landrace	AA	1
486	464	253	Finnish Landrace	AB	1
487	464	382	Finnish Landrace	AA	2
488	312	471	Finnish Landrace	AA	1
489	312	472	Finnish Landrace	AA	1
490	464	293	Finnish Landrace	BB	4
494	464	360	Finnish Landrace	AA	1
506	282	355	Finnish Landrace	AB	4
512	464	401	Finnish Landrace	AB	3
513	378	476	Finnish Landrace	AB	2
514	311	366	Finnish Landrace	AA	4
524	376	477	Finnish Landrace	AA	2
526	376	402	Finnish Landrace	AA	2
527	312	333	Finnish Landrace	AA	2
528	376	478	Finnish Landrace	AA	2
529	312	313	Finnish Landrace	AA	3
530	317	128	Finnish Landrace	AB	2
531	378	403	Finnish Landrace	AA	3
569	412	390	Finnish Landrace	BB	2
570	413	477	Finnish Landrace	AA	2
581	413	416	Finnish Landrace	AB	1
589	412	481	Finnish Landrace	AB	2
593	408	472	Finnish Landrace	AA	1
597	410	419	Finnish Landrace	AA	1
612	408	337	Finnish Landrace	AA	2
614	312	423	Finnish Landrace	AA	2
617	412	485	Finnish Landrace	AA	2
633	413	380	Finnish Landrace	AA	2
645	410	342	Finnish Landrace	AA	1
646	410	488	Finnish Landrace	AA	2
647	410	488	Finnish Landrace	AA	1
235	219	124	Finnish Landrace X Texel cross	AB	1
325	294	296	Finnish Landrace X Texel cross	AA	2
345	294	293	Finnish Landrace X Texel cross	AB	2
346	294	320	Finnish Landrace X Texel cross	AA	2
347	219	321	Finnish Landrace X Texel cross	AA	2
348	219	320	Finnish Landrace X Texel cross	AB	2
351	219	326	Finnish Landrace X Texel cross	AA	1
389	356	158	Finnish Landrace X Texel cross	AA	2
392	356	158	Finnish Landrace X Texel cross	AA	2
431	357	386	Finnish Landrace X Texel cross	AA	3
434	219	387	Finnish Landrace X Texel cross	AA	3
435	219	388	Finnish Landrace X Texel cross	AA	2

437	357	386	Finnish Landrace X Texel cross	AA	1
438	294	389	Finnish Landrace X Texel cross	AA	2
439	294	355	Finnish Landrace X Texel cross	AA	2
441	294	387	Finnish Landrace X Texel cross	AB	2
444	294	391	Finnish Landrace X Texel cross	AA	2
446	294	370	Finnish Landrace X Texel cross	AA	2
448	219	392	Finnish Landrace X Texel cross	AB	3
450	394	322	Finnish Landrace X Texel cross	AA	2
452	394	325	Finnish Landrace X Texel cross	AA	1
453	327	395	Finnish Landrace X Texel cross	AA	3
454	219	396	Finnish Landrace X Texel cross	AA	1
455	394	397	Finnish Landrace X Texel cross	AA	2
456	219	398	Finnish Landrace X Texel cross	BB	2
457	219	393	Finnish Landrace X Texel cross	AA	2
473	357	463	Finnish Landrace X Texel cross	AA	3
475	294	465	Finnish Landrace X Texel cross	AA	1
491	219	462	Finnish Landrace X Texel cross	AA	2
492	294	473	Finnish Landrace X Texel cross	AA	2
493	219	474	Finnish Landrace X Texel cross	AB	1
495	394	475	Finnish Landrace X Texel cross	AA	2
558	219	479	Finnish Landrace X Texel cross	AB	3
561	219	392	Finnish Landrace X Texel cross	AB	0
658	219	345	Finnish Landrace X Texel cross	AB	1
660	219	325	Finnish Landrace X Texel cross	AA	2
662	219	392	Finnish Landrace X Texel cross	AB	2
678	433	434	Finnish Landrace X Texel cross	AB	2
681	219	307	Finnish Landrace X Texel cross	AB	1
685	219	354	Finnish Landrace X Texel cross	AB	2
689	219	389	Finnish Landrace X Texel cross	AB	2
693	219	438	Finnish Landrace X Texel cross	AB	2
703	219	475	Finnish Landrace X Texel cross	AA	1
704	219	365	Finnish Landrace X Texel cross	AB	2
712	433	348	Finnish Landrace X Texel cross	AB	2
748	433	453	Finnish Landrace X Texel cross	AA	1
782	219	475	Finnish Landrace X Texel cross	AB	1
449	219	393	Finnish Landrace X Texel cross	BB	2
198	139	138	composite sheep	AA	2
212	54	198	composite sheep	AA	2
223	115	200	composite sheep	AA	2
229	54	198	composite sheep	AA	2
234	123	218	composite sheep	AA	2
334	113	308	composite sheep	AA	2
373	115	361	composite sheep	AA	2
374	54	362	composite sheep	AA	2
406	113	374	composite sheep	AA	2
497	54	220	composite sheep	AB	3
498	123	198	composite sheep	AA	2
499	115	125	composite sheep	BB	2

502	113	222	composite sheep	AA	2
510	305	224	composite sheep	AA	3
511	113	198	composite sheep	AA	2
515	305	301	composite sheep	AA	2
516	310	330	composite sheep	AA	2
517	305	212	composite sheep	AA	2
518	305	198	composite sheep	AA	2
519	310	223	composite sheep	AA	2
520	305	374	composite sheep	AA	2
522	305	127	composite sheep	AA	2
532	129	226	composite sheep	AA	2
533	227	330	composite sheep	AA	2
534	305	228	composite sheep	AA	3
535	305	130	composite sheep	AB	2
536	227	229	composite sheep	AA	2
537	305	230	composite sheep	AA	2
538	305	404	composite sheep	AA	2
539	305	231	composite sheep	AA	2
540	305	231	composite sheep	AA	2
541	305	131	composite sheep	AA	2
542	305	232	composite sheep	AA	2
543	227	233	composite sheep	AA	1
544	305	215	composite sheep	AA	2
545	227	223	composite sheep	AA	2
546	227	331	composite sheep	AA	2
547	227	121	composite sheep	AA	1
548	227	373	composite sheep	AA	2
549	129	234	composite sheep	AA	1
550	305	405	composite sheep	AA	2
551	305	406	composite sheep	AA	2
552	305	374	composite sheep	AA	1
553	305	334	composite sheep	AA	2
554	305	334	composite sheep	AA	2
555	305	127	composite sheep	AA	1
556	305	127	composite sheep	AA	1
557	394	335	composite sheep	AA	2
766	0	215	composite sheep	AA	1
768	227	198	composite sheep	AA	2
769	0	406	composite sheep	AA	1
770	0	406	composite sheep	AA	1
771	227	515	composite sheep	AA	2
772	227	518	composite sheep	AA	1
773	0	758	composite sheep	AA	2
774	0	545	composite sheep	AA	2
775	0	131	composite sheep	AA	1
776	0	230	composite sheep	AA	1
778	0	542	composite sheep	AB	1
779	0	553	composite sheep	AA	2

References

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