Boyette, Keri Evelyn. Characterization of Follistatin as a Candidate Gene for Litter Size in Pigs. (Under the direction of Dr. Joseph Cassady)

The objective of this study was to characterize follistatin *(*FOL*)* as a candidate gene for litter size in pigs. Litter size is a lowly heritable and sex-limited trait; therefore, response to selection may be enhanced by marker-assisted selection. Our approach for characterizing a region of SSC16, which includes FOL, was to utilize the candidate gene approach using type I and type II markers to determine if FOL had an association with the response to selection for increased litter size in the select line. Pigs genotyped were from a line selected for increased number of fully formed (FF) pigs and a contemporary control line. In generation nine, the estimated breeding value for litter size was 0.63 pigs greater in the select line than in the control line (Holl et al., 2003). A RFLP within *FOL* (n = 251) and the microsatellites, CGT27 (n = 224), S0363 (n=255), S0298 (n=260), and SW1661 (n=253) were genotyped. Effect of marker genotype on FF, number born alive (BA), number still born (SB), and number mummified fetuses (MUM) was tested. Data were analyzed by line with an animal model using MTDFREML. Fixed effects included year and marker genotype. In both the select and control lines, all markers had no significant affect on FF, BA, SB, or MUM when using the animal model. Therefore, follistatin is not likely to have a major effect on litter size in the population studied.

CHARACTERIZATION OF FOLLISTATIN AS A CANDIDATE GENE FOR LITTER SIZE IN PIGS

by

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Chair of Advisory Committee

Personal Biography

Keri Evelyn Boyette was born on June 8, 1981. She is the daughter of Sandra and Ed Boyette and the granddaughter of Aaron and Reba Renfrow and Cleon and Eula Boyette. She has an older brother, Brian and a twin sister, Cheri. Keri grew up in the rural area of Kenly, North Carolina. In 1999, Keri was the salutatorian of her graduating class from North Johnston High School where she was active in many clubs and sports teams. She attended North Carolina State University in the fall of 1999 where she enjoyed working with animals, attending sporting events, and being involved in Campus Crusade for Christ. She received a Bachelor's of Science degree in Animal Science with a minor in Genetics and graduated Magna Cum Laude in the spring of 2003. Keri pursued a Master of Science degree in Animal Science concentrating on Molecular Genetics. She will graduate in December of 2005. She plans to pursue a career in industry working in genetic research.

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Table of Contents

List of Tables

List of Figures

Figure **Page**

Literature Review

Swine Production and Litter Size

Swine production is very important to the economy of North Carolina. Historically, tobacco has been the major farm commodity in the state, but its role as North Carolina's major cash crop has diminished as the nation has learned of harmful effects of tobacco use. Beginning in the 1960's, several forces, including the NC Department of Agriculture, North Carolina State University, and the Agricultural Extension Service, worked to make swine production the replacement activity for tobacco (Swine Odor Task Force, 1995). The state allocated money for swine research, passed laws, and gave tax breaks that facilitated growth of the swine industry. In the 1980's, North Carolina began to increase its pork production, and in the early to mid eighties, the swine population increased by twenty-five percent (NC Department of Agriculture, 1982; 1987). Today, swine production in NC has transformed from being a secondary activity of many farmers to being the focus of large industrial corporations. North Carolina is home to several top pig production firms and is responsible for 16.2% of US production (Furuseth, 1997; NC Department of Agriculture, 2005). It ranks second, behind Iowa, in swine production nationwide and about $1.5 billion is added to North Carolina's economy each year from swine production (NC Department of Agriculture, 2005).

Number of pigs per sow per year is one of the most important factors influencing profitability of pig production. Currently, 43% of the worldwide population consumes pork, making it the major red meat (Rothschild, 2004). Demand for swine has risen, not only as a food source, but also as a model system for human health, causing a need for increased swine production. Foodstuffs are considered a fixed cost for producers. Therefore, increasing litter

size would reduce cost of feed per pig, thus, greatly increasing economic returns with minimal additional inputs (Huges and Varley, 1980; Rothschild, 1996a).

Economical pork production requires that pigs grow fast and efficiently, have high carcass merit and good meat quality, are disease resistant, have high levels of reproductive success, and increased survivability (Rothschild, 2004). The most important requirement of successful swine production is reproductive success, with litter size being the major component of sow productivity. Litter size is expressed as either total number of pigs born or partitioned into components such as number born alive, number of fully formed piglets, number of stillborn piglets, and number of mummies. An ideal gilt would reach puberty earlier in life and would farrow a large litter. She would then return to estrus promptly and be bred successfully for many parities (Rohrer et al., 1999). Given that a sow normally has two litters per year, increasing litter size could make it possible for each sow to have 20-25 saleable pigs each year compared to the current average of 16.9 pigs per year (USDA, Nat. Agr. Stat. Ser., 2005). Currently, a major limit to litter size is uterine capacity (Vallet et al., 2005).

Litter size has many characteristics that influence response to selection. It is a phenotypically variable trait ranging from 2-20 pigs per litter with means around 10 pigs per litter (Linville et al., 2001). According to Blasco and colleagues, the major contributor to variation in reproductive performance in polytocous species such as mice, pigs, and rabbits is prolificacy (1993). Phenotypic standard deviations for litter size are between 2.5 and 3 pigs (Linville et al. 2001). Variation also exists in age at puberty, ranging from 3-7 months of age. Among breeds and populations, there exists significant additive genetic variation (Blasco et al., 1993). Genetic variation in a trait within a population is caused by genetic differences.

Because of variation present in number of pigs born, onset of puberty, as well as genetic variability within breeds and populations, there exists substantial evidence that genetic improvement can be accomplished in regards to litter size (Rothschild and Bidanel, 1998). This improvement has been demonstrated by Johnson et al. (1999) and Holl and Robison (2003).

Much of the genetic variation in litter size is due to it being polygenic in nature. Usually, the number of genes affecting a trait is unknown. In addition, the effect of each gene contributing to the phenotype is not clearly understood (Dekkers, 2003). Like many of the traits that are of agricultural importance, single genes do not have a large enough effect to cause detectable variation, but rather a combination of variation in several genes cause quantitative variation in litter size among animals (Haley and Archibald, 1998).

Traditional selection methods are based on phenotypes. However, in many cases, the phenotype is not expressed or is hard to measure (Soller, 1998). Litter size is a sex-limited trait. Because litter size is expressed only in females, it is hard for producers to determine which males would transmit favorable alleles to their offspring. Litter size is also expressed after sexual maturation in females. Selection of gilts is practiced before they have their first litter. Therefore, producers must select females without knowing whether they carry favorable alleles for large litters.

Another characteristic of litter size that makes selection difficult is that it is lowly heritable. The phenotype of an animal is determined by the genes the animal possesses as well as environmental effects. Heritability is a measure of the strength of the relationship between performance or phenotypic values and breeding values for a trait in a population and ranges in value from 0-1. Low heritabilities indicate that the phenotype or the animal's

performance is not likely to be a good indicator of breeding value. According to Revelle and Robison (1973), small additive genetic variance, extreme levels of environmental variability, negative correlations between direct genetic and maternal effects, or negative genetic correlations between components of the trait may cause the low heritabilities associated with litter size. Current estimates for heritability of litter size range from 0.09-0.14 (Irgang et al., 1994). Low heritability indicates that environment plays a big role in expression of the phenotype.

Changes to environmental factors through management strategies can improve components of litter size very rapidly, but are labor intensive and require continuous inputs, such as improved feedstuffs, vaccines, and housing, and are expensive to implement. Reductions in farrowing interval and pre-weaning mortality have been achieved since the mid 1970's by improving management strategies, but increases in litter size have been nominal (Legault, 1985). On the other hand, genetic improvements are much slower but are permanent, require little maintenance costs, and are passed on to further generations through selection (Archibald and Haley, 1998).

Litter size is a multifactorial trait made up of components such as ovulation rate, uterine capacity, and embryo survival (Gladney et al., 2004). Ovulation rate is defined as the number of ova released while an animal is in estrus (Rohrer et al., 1999). Ovulation rate has an estimated heritability of 0.39, which is higher than that of litter size (Rothschild, 1996a). Uterine capacity is the maximum number of fetuses that the uterus is able to support when not limited by ovulation rate (Christenson et al., 1987). Embryonal survival is the number of embryos that survive and develop into piglets at birth. Heritability is estimated to be 0.30 for embryo survival (Rothschild, 1996a). Because components of litter size tend to have higher

heritabilities than litter size itself, they are often used in quantitative approaches to selection in an index for increased litter size.

Quantitative Approaches to Selection

Fisher's infinitesimal genetic model, which states that a trait is assumed to be determined by an infinite number of genes, each with an infinitesimally small effect, is the basis for quantitative genetic theory (Dekkers and Hospital, 2002). Selection decisions to date have been based on phenotypic data, performance data, or estimated breeding values (EBVs) (Dekkers, 2003). Quantitative genetics involves using population genetic parameters, such as genetic variances, genetic correlations, and heritabilities, for the trait of interest (Dekkers and Hospital, 2002). Phenotypic data from pedigrees are analyzed to estimate genetic parameters, and then the producer determines which animals should contribute to the next generation. According to Rothschild (1996a), over the last few decades, improvements in litter size have been attributed to change in management strategies, use of superior dam lines, and crossbreeding.

Genetic variation for most agriculturally important traits is present within all existing breeds of animals (Soller, 1998). Therefore, when these animals are crossed, hybrid vigor is produced. Because of increased heterozygosity, crossbreeding has been shown to be a successful method to improve reproductive traits. Johnson (1981) noted that improved reproduction was associated with an increase in embryonic survival in crossbred females. A reduced age at puberty, increased conception rates, higher ovulation rates, and increased litter size were observed in crossbred animals (Rothschild and Bidanel, 1998). Therefore, a suggested method to increase litter size in swine is to implement crossbreeding.

Historically, direct selection for increased litter size has been unsuccessful in swine (Ollivier, 1982). However, success has been made in directly selecting for litter size in sheep (Wallace, 1964). Direct selection has also been achieved in the mouse (Falconer, 1971; Gion et al. 1990). Recently, response to selection was accomplished for litter size in swine (Lamberson et al., 1991; Gama and Johnson, 1993; Holl and Robison, 2003). Lamberson and colleagues found success when using tandem selection. First, selection was practiced for high ovulation rate for nine generations, followed by two generations of random selection, and ending with selection for increased litter size for eight generations. They found that selecting for only ovulation rate is not an optimal method to increase litter size in pigs (Lamberson et al., 1991). Johnson and colleagues also saw success when selecting for litter size for three generations after selecting for increased ovulation rate and embryonal survival rate (1999).

In 1973, Revelle and Robison found that selection for increased litter size could be accomplished if an optimal maternal environment was established. Therefore, Holl and Robison (2003) standardized litters to 10 pigs per litter to eliminate the negative environmental correlation that was proven to exist (Falconer, 1960). They directly selected for number born alive for nine generations, and as a result, found that litter size could be increased by direct selection when using an animal model to estimate breeding values (Holl and Robison, 2003). Although success has been made when directly selecting for increased litter size, its lowly heritable nature causes response to selection to be unpredictable from generation to generation unless applied to a large population (Lamberson et al., 1991). A more consistent response to selection could be achieved if direct selection could be applied on both genders through use of molecular techniques (Linville et al., 2001).

Since response to direct selection for litter size has been inconsistent over the years, and heritabilities of litter size components tend to be higher than litter size itself, components have been used to select for litter size indirectly by using a selection index (Rothschild and Bidanel, 1998). Using an index of selection, which includes components such as ovulation rate, embryonal survival, or uterine capacity, is suggested to yield a greater response than directly selecting for litter size (Bennett and Leymaster, 1989). Using an index requires that phenotypic measurements are chosen to estimate genetic merit for the selection objective.

Bennett and Leymaster (1990b) found response to selection for an index including ovulation rate and uterine capacity was 13% greater than an index based on ovulation rate and embryonal survival. Response to selection for the index, which included ovulation rate and uterine capacity, was 27% greater than from direct selection for litter size (Bennett and Leymaster, 1990b). Quantitative approaches to selection have caused great rates of genetic improvement, but they still have several limitations including inability to determine favorable alleles from phenotypes, expression of some traits being sex-limited or expressed later in life, and the lowly heritable nature of reproductive traits (Dekkers and Hospital, 2002). Use of molecular genetics may help improve response to selection for increased litter size.

Molecular Techniques

Genetic evaluation using quantitative approaches typically involves identifying genetic influences by analyzing phenotypic values, while molecular approaches examine alleles at the DNA level and relate them to phenotypes (Beuzen et al., 2000). Identifying quantitative trait loci (QTL) is one of the main goals of molecular technology in animal breeding. A QTL is a location on a chromosome that contains a gene affecting a quantitative trait. Molecular technology has the ability to be used to reduce some of the limitations found

when using quantitative methods. Advancement of biotechnology has made it possible to examine genetic variation at the molecular level. Therefore, the exact proportion of variation can be determined instead of relying on estimates from phenotypic data using quantitative genetics (Visscher and Haley, 1995).

Molecular technology uses molecular markers to determine if there is an association between a chromosomal region and expression of a particular trait (Haley and Archibald, 1998). Genetic markers that represent a certain gene and function are known as type I markers. These markers usually encode a particular peptide sequence. A second type of marker, known as a type II marker, is one that contains large amounts of variation and consists of DNA sequences with no known function (Haley and Archibald, 1998).

Type I markers are based on a particular gene and usually contain polymorphisms. Polymorphisms are genetic variants at the molecular level. There are two categories of polymorphisms: those that contain base-pair substitutions or small structural changes consisting of additions, deletions, or inversions, and those that contain variable number of tandem repeats of short motifs (Soller, 1998). Polymorphisms are abundant throughout the genome and can provide markers near or in the gene of interest (Beuzen et al., 2000). Single nucleotide polymorphisms (SNPs) involve substitution of one nucleotide for another and can be located in the coding region of a gene and can therefore affect protein function. SNPs are usually inherited more stably than microsatellite markers and are also more suitable for high throughput technology such as microarrays (Lipshutz et al., 1999).

Microsatellite markers are the most common type II markers. They are found in high number and at random in the genome, which makes them a great tool in molecular genetics (Haley and Archibald, 1998). Eukaryotes are estimated to have tens of thousands

microsatellites throughout their genome (Hamada et al., 1982). Many times these are found in introns, or non-coding regions (Beuzen et al., 2000). Their high degree of heterozygosity makes them ideal markers for genetic studies (Callen et al., 1993). Microsatellites usually consist of dinucleotide repeats, with the dinucleotide repeated about ten times. The most common dinucleotide repeat in mammals is $(CA)_n$ (Beuzen et al., 2000). Oligoprimers flank dinucleotide repeats. Therefore, when a mutation occurs within the DNA sequence complementary to the oligoprimer, it may prevent primers from binding correctly and cause reduced amplification or loss of PCR product (Callen et al., 1993). Mutation rate of microsatellites is considered high, and therefore, they are considered to be good markers in molecular technology because there tends to be large numbers of alleles that vary in size at each locus. Variation in microsatellite length tends to be generated by DNA polymerase slippage and mismatch repair during replication (Beuzen et al., 2000). When microsatellite markers are chosen for an association study, markers that contain null alleles must be avoided. Null alleles occur when PCR amplification is completely prevented and no PCR product is formed for one allele. If null alleles go undetected, misleading data may be generated when determining if linkage disequilibrium exists between a DNA sequence and the trait gene (Callen et al., 1993). Microsatellites are useful in molecular genetics because of variation in the number of times the sequence is repeated (Montaldo and Meza-Herrera, 1998). Both type I and type II markers are used to follow inheritance patterns of segments of chromosomes from parents to offspring and to compare expression of those regions to the phenotype expressed (Visscher and Haley, 1995).

Molecular Marker Approach

The aim of genomic analyses today is to find genes or quantitative trait loci that are responsible for variation in traits of economical importance in livestock (Rothschild and Soller, 1997). In order to get a fundamental understanding of genes involved in a quantitative trait, location of markers linked to that trait need to be identified. There are two common methods for showing an association between a gene and a trait. They are known as the molecular marker approach and the candidate gene approach. Both of these methods use molecular markers to show that inheritance of a gene or chromosomal region is linked with variation in performance (Haley and Archibald, 1998).

The molecular marker approach is also known as linkage mapping or positional cloning. It consists of three main steps including scanning the entire genome of a species with evenly spaced, highly polymorphic markers, calculating a linkage statistic at each position along the genome, and identifying regions that show a significant deviation from the expectation of independent assortment (Lander and Kruglyak, 1995; Kwon et al., 2000). The scan can be done on an individual chromosome or the entire genome. Tracking inheritance of genetic regions throughout the genome in pigs would require about 100-150 evenly spaced markers (Haley and Archibald, 1998). Number of markers required for a chromosomal scan depends on chromosome length. Marker positions are usually estimated. Genome scans identify chromosomal regions that affect a trait by using markers that are normally 5-10 cM apart. In most species, a centimorgan (cM) is about 500kb-1Mb (Dekkers and Hospital, 2002). Genome scans are one of the most complete methods for searching for QTLs across the genome (Wilkie et al., 1999). With the discovery of microsatellite markers, a geneticist

can now cover the entire genome with markers about 10 cM apart and fine-map a trait gene in about a year (Stephen et al., 1994).

Marker location is very important when doing a genome scan because genes that are close to each other on a chromosome do not assort independently during meiosis. Genes that are far apart on a chromosome are subject to recombination and therefore, do appear to assort independently. Marker inheritance is followed to find an association between a chromosomal region and trait variation in a large population (Archibald and Haley, 1998). Geneticists are able to identify genetic regions that are linked to a trait by observing marker variants, common in a particular population, occurring more frequently than would be expected by chance (Kwon et al., 2000). If there is a significant association between chromosomal region and the trait, the chromosomal region likely contains a gene or genes that affect the trait of interest (Archibald and Haley, 1998).

There are advantages and disadvantages of performing a genome scan instead of using a candidate-gene approach. One advantage is that the molecular scan can detect any loci with large effects on a trait. Loci do not have to be acknowledged as genes in order to be discovered (Haley and Archibald, 1998). Genes involved in genetic architecture of traits may be stumbled upon when using the genomic scan approach since knowledge of physiology or biology of the trait is also not required to perform a scan (Kwon et al., 2000). Another advantage of the genome scan over a candidate-gene approach is that most quantitative traits are affected by many genes with small effects. Because of their complex genetic basis, allelic variation at a single locus rarely describes the studied phenotype (Lander and Schork, 1994).

One disadvantage of genome scans is that they require a well designed population, and a large number of genotypes (Wilkie et al., 1999). Individuals under study must be

related so that inheritance can be tracked (Kwon et al., 2000). In order to get a good coverage of the genome, a large number of animals must be genotyped (Haley and Archibald, 1998). Genome scans locate regions that are linked to expression of a trait. Therefore, after this region is located, more research needs to be done to find exactly what gene is affecting the trait. Therefore, this method can be very time consuming and will generate large amounts of data due to the large number of genotypes required.

The molecular marker method has been successful in identifying regions of chromosomes that affect quantitative traits. QTL for litter size have been identified on several chromosomes including chromosomes 6 (Wilkie et al., 1999), 7 (Rothschild and Plastow, 1999), 8 (King et al., 2003), and 11 (Cassady et al., 2001). QTL for uterine capacity, a component of litter size, has also been identified on chromosome 8 (Rohrer et al., 1999) and 2 (Fahrenkrug et al., 2000).

Candidate-Gene Approach

The candidate-gene approach is the most direct method of testing relationships between a gene and a phenotype (Streelman and Kocher, 2000). The candidate-gene approach can be utilized in any populations in which phenotypes can be measured (Rothschild and Soller, 1997). Whereas genome scans will find associations between a gene and a trait without a sufficient knowledge of the gene, the candidate-gene approach requires that the physiological basis of the trait is understood in order to pick informative candidate genes (Rothschild, 1998).

The first step of the candidate-gene approach is to find a suitable candidate gene (Streelman and Kocher, 2000). A candidate gene is a sequenced gene that is known to be involved in development or physiology of the trait (Bryne and McMullen, 1996). Basis of the

candidate-gene approach is to look at variation within a trait at the molecular level in known genes that are thought to play a role in expression of the trait (Soller, 1998). Therefore, a basic understanding of biology and physiology of the gene and trait is required in order to choose the most likely candidate gene (Streelman and Kocher, 2000; Kwon et al., 2000). Candidate genes may also be chosen based on effects seen from knocking out genes in a model species, such as a mouse (Rothschild and Soller, 1997) or by comparative mapping of a gene in another species.

Since the candidate-gene approach focuses on polymorphisms within the gene, the next step of this approach is to determine which polymorphisms would be most informative in an association study (Streelman and Kocher, 2000). The pig genome consists of numerous polymorphisms that are likely to exist every few hundred base pairs and may be located within the intron or exon of the candidate gene. The candidate-gene approach looks for a relationship between the polymorphism within or near the gene and phenotypic expression of a trait. An association exists only if the polymorphism is in linkage disequilibrium with the causative region of the observed phenotype (Archibald and Haley, 1998). Once an association is found between the gene and trait of interest, selection would be practiced on the gene variant that would positively influence the trait. Through an analysis of variance, association between the DNA structural variants and value of the trait will be determined (Soller, 1998). Since the candidate-gene approach focuses on polymorphisms that are hypothesized to affect a trait, markers are often found to be very closely linked to QTL (Dekkers and Hospital, 2002).

Other populations may not have the same association between a candidate-gene and trait as the population studied or the association may be limited to the specific generation

studied. This outcome may be due to recombination events, absence of analogous segregating QTL nearby, absence of informative markers, or sampling (Vallet et al., 2005). When a candidate-gene association is discovered in a particular population, the next step is to confirm existence in subsequent generations and in a different population. Future studies should be conducted in populations consisting of the same breed as well as other breeds to add power in the association study (Rothschild and Soller, 1997). Polymorphisms that hinder gene function would be expected to also cause similar results in other populations (Vallet et al., 2005). After the association is shown to exist in other populations, a DNA-based test could be created that would identify animals carrying favorable alleles and would improve the chances of selecting valuable animals (Haley and Archibald, 1998).

Some advantages of the candidate gene approach include that it requires less animals than the genome scan, can be conducted in one generation, and can be performed on unrelated animals (Archibald and Haley, 1998; Kwon et al., 2000). It can also be used to compare differences in a control and select group (Kwon et al., 2000). Unlike the genome scan approach, complete pedigree information from multiple generations is not required on animals being studied (Rothschild and Soller, 1997). It is also attractive to many geneticists because it has potential to quickly identify linkage between a marker and a QTL (Soller, 1994). The candidate-gene approach is relatively easy to implement and positive results can easily be incorporated into breeding schemes with the use of marker-assisted selection (MAS) (Soller, 1998).

The polygenic nature of quantitative traits limits use of the candidate-gene approach (Montaldo and Meza-Herrera, 1998). Because quantitative traits are influenced by many genes with small effects, many associations might be overlooked using this method.

Currently, only a small proportion of genes that influence phenotypic traits are known, making the choice of a candidate gene difficult. Once a gene is chosen, it is difficult to discern if the observed trait differences are actually caused by the candidate gene or by other genes in the immediate area. Therefore, fine mapping is required to focus on the actual gene affecting a trait. Another disadvantage of the candidate-gene approach is that it tends to have high initial start up costs due to the development of primers and identification of polymorphic sites (Rothschild and Soller, 1997).

Because of the slow rate of genetic improvement observed in reproductive traits, many scientists have begun looking at single genes that may affect reproduction (Rothschild, 1996b). Use of the candidate-gene approach for reproductive traits has been successful in pigs. The estrogen receptor gene (ESR) has been demonstrated to have an association with litter size using this approach. Allelic affects of the ESR gene are quite large, ranging from 0.4 to 1.15 pigs per litter (Rothschild et al., 1996b; Short et al., 1997). More recently, prolactin receptor and erythropoietin receptor have also been shown to be associated with litter size in swine (Vincent et al., 1998; Vallet et al., 2005). However, Vallet and colleagues did not use an animal model during data analysis, which may result in spurious results due to an underestimation of standard errors.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is used to amplify regions of DNA. Kary Mullis created PCR in 1984. He later received the Nobel Prize for his accomplishment in 1993 (Snustad and Simmons, 2000). PCR leads to an exponential increase in the number of copies of a target sequence by repeating the major steps for 30-40 cycles.

It involves using primers explicitly designed for the gene of interest, individual nucleotides, a polymerase, and a stabilizing buffer. The three main steps of PCR are denaturation, annealing, and extension.

Denaturation usually occurs around 94-97ºC. During this step, genomic DNA containing the region to be amplified is denatured into single strands. All enzymatic reactions stop during this step of PCR. During the annealing stage, which occurs around 50-60°C, denatured DNA anneals, or adheres, to the primers. Primers are made of specific sequences and therefore bind to a specific location on the DNA. Therefore, alleles of the specific marker of interest are amplified from the DNA sample of an individual animal through use of specific primers (Buratowski, 1994; Koleske and Young, 1995, Stein et al., 1996; Haley and Archibald, 1998). Each set of primers has an optimal temperature for which this step occurs that must be determined prior to PCR. The last step of PCR is extension, which occurs around 70-72°C. During this phase, DNA polymerase is used to replicate the DNA segment, using free nucleotides, between sites complementary to the primers. The DNA provides template, while the primer provides the 3'OH required for covalent extension (Snustad and Simmons, 2000). The steps are repeated about 30-40 times, and the end result is an exponential number of copies of the gene of interest.

Restriction Fragment Length Polymorphism (RFLP)

One of the oldest methods of typing polymorphisms in a population is through restriction fragment length polymorphism (RFLP). RFLP technology uses restriction endonucleases that recognize and cut at certain sequences called recognition sites (Beuzen et al., 2000). The polymorphism in the DNA sequence, which can be single base pair polymorphisms or insertions or deletions of blocks of DNA, causes the restriction enzyme to

yield fragments of various lengths that can be visualized on an agarose gel (Botstein et al., 1980). An electric current is applied to the gel and the DNA fragments are separated based on their molecular size. Smaller fragments move faster through the gel while larger fragments migrate slower. If the nucleotide change alters the restriction enzyme recognition site, the DNA sequence either gains or loses ability to be cleaved at that site. A long fragment is generated when the restriction site is absent, while two shorter fragments are generated when the restriction enzyme has the ability to cleave (Beuzen et al., 2000).

RFLP is not convenient for high throughput studies due to its gel-based approach. It is very labor intensive in the development and typing stages (Dodgson et al., 1997). Another disadvantage of this method is that most mutations do not alter recognition sites and would be overlooked by a RFLP analysis (Beuzen et al., 2000).

Genetic Linkage Maps

Detection of QTL using genetic markers requires development of a genetic map of the species of interest (Bovenhuis et al., 1997). Currently, there are three types of genetic maps that give a schematic representation of DNA including physical maps, genetic maps, and cytogenetic maps. Development of a genetic map requires access to pedigree information with known relationship data and a population having polymorphic loci (Rothschild, 1998). Mapping of genes is based on recombination frequencies and genetic marker genotyping in a reference population. Many laboratories are collaborating worldwide in order to produce the most informative swine genetic map (Beattie, 1994). The last 10 years has seen a huge increase in the number of mapped genes in the pig. In 1989, there were only 50 genes and markers (Rothschild and Plastow, 1999). Today there are over 3500 markers that can be used to track inheritance of a particular trait (Rohrer, 2004). Success seen in the last few years is

partially attributed to the high level of conservation of gene sequences between humans, cattle, sheep, goats, pigs, and mice (Montaldo and Meza-Herrera, 1998). Because of the high level of conservation between species, comparative mapping experiments can be done to fill in gaps of the species of choice. Association studies have been greatly enhanced by improvement of genetic maps so that now there are thousands of markers available for association studies.

Genotyping

Development of molecular markers has made genotyping possible with use of evenly spaced polymorphic microsatellite markers (Visscher and Haley, 1995). Technology has also improved genotyping methods through advances in robotic devices, PCR multiplexing methods, fluorescent detection systems, software for fragment size analysis and data tracking, and availability of thousands of microsatellite markers (Idury and Cardon, 1997). Microsatellite markers contain sequence repeats, usually made of dinucleotides, consisting of $(AC)_n$ repeats. Microsatellite markers are PCR based, and therefore, only small quantities of genomic DNA is necessary for genotyping (Perlin et al., 1994). Large numbers of samples can be rapidly genotyped through the advancement of technology.

Automated machines have allowed high-throughput genotyping through use of a capillary system. The capillary is a small, flexible tube that contains polymer. Polymer allows DNA samples to electrically migrate from one capillary end to the other. Markers that are fluorescently labeled using one of three dye sets are used in PCR, which allows several markers to be genotyped at once through multiplexing. The dyes fluoresce at different wavelengths, and therefore, alleles can be distinguished even when PCR products overlap (Zielge et al., 1992). When electrical current is applied to the samples, negatively charged

DNA migrates through the capillary toward the electrode and past a detector window where a laser excites the fluorescent dye. The CCD camera then translates the fluorescent intensity into data that is sent to a file on a computer. Software programs have been developed that read intensity of the dye as well as intensity of a size standard. Sample size is then estimated from the size standard in an analysis software program.

Automated fragment analysis is an improved method over older radioactive labeling methods for genotyping. Genotyping with use of highly polymorphic loci has limitations in scoring accuracy and genotyping efficiency (Ziegle et al., 1992). Decreases in accuracy may be caused by incorrect allele sizing due to indistinct peaks in electropherograms, misplacement of PCR products when preparing a plate for loading, as well as DNA sample contamination (Li et al., 2001). Limitations are reduced through use of fluorescently labeled markers and automated fragment analysis. Automated fragment software provides an electropherogram of the genotype call that can distinguish between homozygotes and heterozygotes differing by only two base pairs. It can also analyze markers that vary from 2-9 repeated nucleotides. Another improvement over older methods is that numerous markers can be multiplexed. There is a three-fold improvement in the number of markers that can be genotyped in a single lane because of available dyes. The biggest advantage of this method is that all marker data are electronically submitted into a computer database, which allows higher throughput and less time genotyping animals (Ziegle, 1992). Before automated fragment equipment was used, alleles were visually interpreted and then entered into a database which was very time consuming and open to higher rates of human error (Perlin et al., 1994). Using automated fragment analyses, molecular data are automatically entered into a database and can easily be accessed for genetic analysis.

Data Analysis

A number of studies over the past few years have looked at effects of single genes on a trait of interest and have found significant results indicating that a single gene is associated with a quantitative trait of economic importance (Kennedy et al., 1992). In order for a marker to be implemented into a breeding program using MAS, an analysis designed to detect QTL is performed (Dekkers and Hospital, 2002). Data in an association study are normally either analyzed using a general linear regression model or a mixed model. To date, most studies have used a general linear regression model without realization that these types of analyses tend to ignore relationships among individuals due to polygenes. A correlation exists between data from related individuals (Haley and Archibald, 1998). When these relationships are ignored, this type of analysis can lead to an increase in false significant effects no matter if selection has or has not been practiced. Spurious results are caused by an underestimation of standard errors that are associated with genotypic effects and lead to an increased probability of a type I error (Kennedy et al., 1992; Allen-Brady et al., 2003). This is particularly true if data from several generations are used when looking for an association. Kennedy and colleagues (1992) found that when selection was practiced for at least five years, an incorrect association was seen between a gene and a trait 29% of the time. Therefore, a more efficient model is required.

The suggested analysis to overcome problems seen in the general linear regression model is to use a mixed-model procedure under the animal model. This type of model accounts for all known relationships between individuals by using pedigree information. The suggested method to determine gene effects is to include genotype as a fixed effect in the

model. This approach would allow effects from single genes and polygenes to be separated and would therefore properly estimate standard errors (Kennedy et al., 1992).

Marker-Assisted Selection

Despite advancements made in molecular genetics through understanding of gene structure and expression, it has currently made little contribution to animal breeding (Beuzen et al., 2000). Selection decisions are sometimes difficult to make when using quantitative approaches that are based on phenotypes because animals that carry favorable alleles for a trait may not be distinguishable from those that do not. Despite its lack of use in breeding programs, molecular data have the ability to influence selection decisions through marker-assisted selection (MAS). Marker-assisted selection uses DNA sequences that are associated with a specific trait to supplement phenotypic data used in the quantitative approach to selection (Parmentier et al., 2001).

Advancements in molecular technology have allowed marker information to be utilized in selection decisions (Southwood et al., 1998). There are three phases that must be implemented in order for MAS to be used in a breeding program. In the detection phase, DNA polymorphisms are used as markers to search for QTL affecting a trait. Markers that are linked to the QTL are then identified as well as their location and association with the trait. In the evaluation phase, markers linked to QTL are tested in other populations to determine whether the QTL is also segregating in the target population. Once proven to segregate in other populations, tests can be developed and utilized on animals to determine whether they carry favorable alleles (Davis and Denise, 1998). Short and colleagues (1997) report that Pig Improvement Company (PIC) is using MAS to select for litter size using the estrogen receptor gene. However, these tests can be expensive to develop and therefore, the

average breeder cannot afford to use it in their selection program. Consequently, molecular data are more commonly used to supplement quantitative data. The third phase in MAS is implementation. After a QTL is identified and its effects are estimated, data from traits are combined into a molecular score. Molecular score has the potential to be used in selection systems through best linear unbiased predictor (BLUP). Hence, marker information can be added to equations involving the mixed model to determine animals selected for breeding programs (Kinghorn and Clarke, 1997).

Through MAS, animals that are carrying favorable alleles at a QTL can be selected based on a direct evaluation of their molecular biology (Soller, 1998). Marker-assisted selection is best implemented for traits that are lowly heritable, expressed early in life, and sex-limited and therefore, can cause genetic improvement in the population (Lande and Thompson, 1990; Soller, 1994; Southwood et al., 1998). MAS is also most effective for traits that are affected by a small number of genes with large effects rather than with many genes with small effects (Montaldo and Mesa-Herrera, 1998; Wilkie et al., 1999). Use of MAS requires that recombination rates between markers and genes of interest are low. Thus, the marker must be located in close proximity to the gene so that they will not assort independently during meiosis (Archibald and Haley, 1998).

Marker-assisted selection can improve accuracy of selection. By identifying small numbers of genes with large affects, marker-assisted selection has the ability to improve selection accuracy (Rathje et al., 1996; Rothschild et al., 1996). Selection accuracy is a measure of the correlation between the predicted value and true breeding value. Despite genotyping errors, molecular techniques are subject to less environmental factors and therefore, tend to be more accurate than traditional selection methods. Selection accuracy is

also improved because it allows identification of favorable alleles in both males and females (Soller, 1998; Drogemuller et al., 2001). Marker-assisted selection can also be implemented early in life so that breeders do not have to wait for animals to phenotypically express the trait.

Marker-assisted selection will be used more often in future selection programs when more associations between markers and traits are identified (Rothschild, 1998). For reproductive traits such as litter size, MAS is a promising technique that may aid in genetic improvement due to its low heritability and availability of genetic markers (Southwood et al., 1998; Drogemuller et al., 2001). Currently selection must be based on a combination of markers along with phenotypic data because genetic markers do not explain most of the genetic variation of any trait (Dekkers and Hospital, 2002).

Follistatin

Reproduction is economically important to the swine industry and is controlled by numerous genes. Number of genes affecting a trait is usually unknown. To gain a better understanding of such complex traits, scientists have begun to look for single genes that may have an effect. Currently, scientists are looking for single genes that affect litter size in swine (Dekkers, 2003; Rothschild, 1996b). Research has shown that hypothalamic, pituitary, and gonadal hormones usually play a role in reproductive traits (Igarashi et al., 1993). Increased proliferation has been associated with growth and development of primordial follicles up to the ovulatory stage (Knight and Glister, 2001a). There has also been evidence that locally produced growth factors act as co-regulators of folliculogenesis (Knight and Glister, 2001b). Some growth factors that are thought to be associated with reproduction are inhibin, activin, and follistatin. They were first identified in ovarian follicular fluid in the mid 1980's (Patel,

1998). Activins and inhibins are both part of the TGF-β superfamily of proteins and are structurally similar (Phillips, 2005). Follistatin is structurally unrelated to activin and inhibin, but is functionally linked through its binding ability (Knight and Glister, 2001a; Phillips, 2005). Together, all three function to regulate production of follicle stimulating hormone (FSH).

Existence of inhibin was first suggested in 1923 when it was detected in testicular extracts and thought to regulate pituitary activity (Phillips, 2005). It was not until 1983 that it was discovered in ovarian follicular fluid (Igarashi et al., 1993). Inhibin is found in the pituitary and gonads and functions as a reproductive hormone to enhance theca cell production of LH-induced androgen. It may also be involved in production of progesterone by luteal cells (Knight and Glister, 2001b). It also inhibits production of FSH (Knight and Glister, 2001a). Inhibin contains a heterodimeric α:β-subunit (Muttukrishna and Ledger, 2001). There are two types of β subunits that make up inhibin and both act on the pituitary to decrease release of FSH (Ying, 1988; Cataldo et al., 1994). Activin is very similar structurally to inhibin. It is made of β:β dimers linked with disulphide bonds and weighs about 25kDa (Muttukrishna et al., 2004). All reproductive tissues contain activin β-subunits (Matzuk et al., 1995a). Activin is also involved in the pituitary and gonads as well as a large variety of organs such as the CNS, bone marrow, and pancreas (Igarashi et al., 1993). Activin stimulates granulosa cell proliferation and differentiation and also promotes synthesis and release of FSH (Igarashi, 1993; Knight and Glister, 2001a; Muttukrishna, 2004; Gladney et al., 2004). It is also involved in folliculogenesis. Activin contains receptors known as ActRcII that are necessary for folliculogenesis to occur. If interaction with type II receptors is prevented by follistatin, folliculogenesis will not occur (Matzuk et al., 1995; Shimonaka et

al., 1991). Matzuk and colleagues looked at the role of ActRcII in the activin-signaling pathway affecting reproduction in both males and females (1995a). Using embryonic stem cell lines, mice were created in which the ActRcII gene was knocked out. Male mice that were deficient in ActRcII were delayed in reaching fertility and had smaller testes until adult stage. However, spermatogenesis was unaffected. Affects of ActRcII deficiency were more dramatic in females. Those that were lacking ActRcII had thinner uteri, smaller ovaries, and increased follicular atresia. Therefore, females did not have normal estrous cycles and were infertile. As shown in mice, the activin-signaling pathway is very important to reproductive activity.

Follistatin is encoded by a single gene and is a cysteine rich monomeric polypeptide that is composed of about 36 cysteine residues (Patel, 1998; Muttukrishna et al., 2004). The follistatin protein varies in size from 32-39kDa and the gene is mapped to SSC16 (Muttukrishna et al., 2004; Ellegren, 1993). Follistatin is well conserved across mammalian species with 95% homology. It contains a region encoding for a short signal peptide and 5 other exons. It is subject to alternative splicing, which normally takes place at the 3' end of the gene, and has the option of keeping exon 5. Exon 5 is retained in the most abundant species of follistatin (Patel, 1998). Two major splice variants of follistatin are FS315 and FS288, consisting of 315 and 288 amino acids, respectively. Both forms have a high affinity for binding activin, but FS288 also binds heparin with a high affinity (Muttukrishna et al., 2004). Because of its high affinity for heparin, FS288 is associated with cell surfaces. FS315 is the major form that is found in circulation but can be altered to create a form containing only 300 amino acids that is abundant in ovarian follicular fluid (Phillips, 2003). Follistatin is

also subject to post-translational modifications including glycosylation and proteolysis (Patel, 1998; Knight and Glister, 2001a).

Follistatin is most abundant in pituitary glands, gonads, and kidney, but can be found in any tissues where activin is present (Michel, 1993). Follistatin has the ability to bind to many members of the TGF-β family of proteins, thus neutralizing many of their actions (Patel, 1998). Follistatin is structurally unrelated to activin and inhibin but binds to their β-subunits (Phillips and Krester, 1998). It has a great affinity for binding activin, and to a much lesser extent, inhibin. Because of the β-subunits, two molecules of follistatin can bind to activin, while only one molecule can bind to inhibin (Shimonaka et al., 1991). Binding of both β-subunits causes follistatin to prevent activin's interaction with its receptors and thus modulates production of activin in granulosa cells (Cataldo et al., 1994). Absence of follistatin allows activins to promote synthesis of FSH while also inhibiting secretion of growth hormone (GH), prolactin (PRL), and adrenocorticotropic hormone (ACTH). Under these circumstances, the animal enters into a reproductive phase (Patel, 1998). High levels of follistatin cause it to bind to activin, which blocks interactions of activin with receptors on target cells and prevents activin from acting on other tissues (Knight and Glister, 2001b). Once follistatin binds to activin, levels of free activin decline. This binding causes inhibition of synthesis of FSH as well as release of GH, PRL, and ACTH. Therefore, the animal enters a non-reproductive state (Patel, 1998). Follistatin also binds to inhibin, but with a much lower affinity. Binding of follistatin does not seem to decrease inhibin activity like it does activin (Knight and Glister, 2001b).

Follistatin is a protein that is essential for life. If not present, animals will die shortly after birth (Matzuk et al., 1995b). Mice have been used to study effects of follistatin. In mice,

follistatin can be detected as early as day 5.5 while in the embryonic stage. It can later be detected in developing hindbrain, vibrissae, teeth, epidermis, and muscle (Guo et al., 1998). Matzuk and colleagues did an experiment to define the role of follistatin in mammalian development (1995b). They created knockout mice that were lacking the 6-exon follistatin gene. Then mice that were heterozygous for the mutation were crossed so that mice homozygous for the follistatin deletion were created. Mice were genotyped at embryonic day 18.5 and at birth. Genotyping data showed that animals deficient for follistatin could survive until shortly after birth. Those that were deficient in follistatin also grew slower than those with follistatin, had shiny, tight skin, delayed incisor development, decreased muscle mass, skeletal abnormalities, were lacking a hard palate, and were smaller than their littermates. Follistatin deficient mice also looked pale at birth, and when dissected, the lungs sank in liquid and had poorly developed alveoli. This outcome partly explains why mice deficient in follistatin have trouble breathing and die shortly after birth. Because mutant mice died, the role of follistatin in adult reproduction could not be deducted. The wide variety of birth defects in follistatin deficient mice shows the range of functions in which follistatin is involved during development.

In 1998, Guo and colleagues wanted to further study effects of follistatin during reproduction and development in mammals. They produced transgenic mice that over expressed follistatin. The transgene was expressed in a variety of tissues. Mice that were overexpressing follistatin developed normally, and no deleterious effects were discovered. One visible difference in mice overexpressing follistatin was that they had shiny, irregular fur (Guo et al., 1998). This study also showed that follistatin affects reproduction. Reduced fertility was observed in mice that overexpressed follistatin. Reduction in fertility correlated

with amount of overexpression. Overexpression of follistatin resulted in males having smaller testes and females having thinner uteri, showing that follistatin is clearly important to sexual development.

In 2004, Jorgez and colleagues also studied the effects of follistatin on reproduction. Because follistatin null mice die shortly after birth, a line of mice was produced to cause the follistatin gene to become null specifically in granulosa cells. Mice with the follistatin gene deleted in their granulosa cells showed a reduction in litter size and number of litters per month. Some mice were infertile. Histological examinations showed that number of follicles was drastically reduced by six months of age (Jorgez et al., 2004).

Effects of follistatin have also been studied in pigs. In 2004, Gladney and colleagues evaluated the gene expression of follistatin in porcine ovarian follicles between a select and control line undergoing selection for sixteen generations. The select line had been selected for an index of ovulation rate, embryo survival, and litter size, while the control line was randomly selected. Differential display PCR (ddPCR) and DNA microarray analyses were implemented to study gene expression differences between the two lines. Microarray analysis demonstrated differences in follistatin gene expression between the two lines. Northern hybridization confirmed the microarray results. Allelic variation caused by selection or transcriptional changes in other genes that interact with reproductive QTL may explain the differences in gene expression between the two lines (Gladney et al., 2004). However, more research is needed to determine other factors influencing follistatin expression in swine. It can be concluded from these four studies that follistatin is important in reproduction.

Follistatin is not only involved in reproduction. It has been shown to be involved in inflammatory responses. Studies were performed on sheep that had undergone surgery.

Follistatin levels rose in response to surgical stress (Klein et al., 1993). The mechanism of follistatin is not fully understood. However, when sheep were challenged with interleukins, follistatin levels also increased, demonstrating that they are somehow linked to the acute phase response (de Krester et al., 1999).

Efforts are being made to create assays that will measure amounts of inhibin, activin, and follistatin in various tissues. Assays have been developed to measure the three proteins in humans. Activin A is highly conserved among species at the protein level. Therefore, human activin A ELISA has been utilized in a number of species including sheep, cow, rat, and mouse. To date, the human inhibin and follistatin assays have not been practical for other species (Phillips, 2005). This is partly due to the differing structures of the molecules. Human follistatin contains a unique region made of 171-175 amino acids (Evans and Groome, 2001). Therefore, the human follistatin assays will not cross-react with other species. This problem needs to be overcome, as well as increasing the accuracy with which the three forms of follistatin are measured. Currently, the human assay cannot distinguish between the three forms of follistatin and so accuracy of measuring follistatin levels is low.

Summary

Pork is essential to North Carolina's economy and is a popular worldwide meat source. Litter size is an important trait in the swine industry and can be increased without causing the producer additional inputs. An additional pig provides a larger profit for the producer, which drives the industry to improve the trait.

Traditional methods of selection for litter size include using phenotypic data, which includes all genes and environmental factors, to create breeding values for each animal. Breeding values are then used to make selection decisions of which animals will contribute

favorable alleles to future generations. Some strategies including improvements to management practices, crossbreeding, and use of superior dam lines have been used to increase litter size. However, limitations exist using these methods because litter size is lowly heritable, sex-limited, late-onset, and polygenic. These limitations may be decreased if animals could be genotyped earlier in life using molecular techniques to directly identify which alleles they possess. Molecular technology can also identify QTL that can be incorporated into breeding programs through marker-assisted selection.

Recently, researchers are searching for candidate-genes that affect traits. One likely candidate-gene for litter size is follistatin. Research shows that follistatin is involved in reproduction through its interaction with other reproductive hormones. Based on its effects in mice, follistatin may affect reproduction in pigs. The objective of this research is to determine if follistatin is a likely candidate gene for litter size in swine.

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CHARACTERIZATION OF FOLLISTATIN AS A CANDIDATE GENE FOR LITTER SIZE IN PIGS

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Abstract

The objective of this study was to characterize follistatin *(*FOL*)* as a candidate gene for litter size in pigs. Litter size is a lowly heritable and sex-limited trait; therefore, response to selection may be enhanced by marker-assisted selection. Our approach for characterizing a region of SSC16, which includes FOL, was to utilize the candidate gene approach using type I and type II markers to determine if FOL had an association with the response to selection for increased litter size in the select line. Pigs genotyped were from a line selected for increased number of fully formed (FF) pigs and a contemporary control line. In generation nine, the estimated breeding value for litter size was 0.63 pigs greater in the select line than in the control line (Holl et al., 2003). A RFLP within *FOL* (n = 251) and the microsatellites, CGT27 (n = 224), S0363 (n=255), S0298 (n=260), and SW1661 (n=253) were genotyped. Effect of marker genotype on FF, number born alive (BA), number still born (SB), and number mummified fetuses (MUM) was tested. Data were analyzed by line with an animal model using MTDFREML. Fixed effects included year and marker genotype. In both the select and control lines, all markers had no significant affect on FF, BA, SB, or MUM when using the animal model. Therefore, follistatin is not likely to have a major effect on litter size in the population studied.

Introduction

The pig is an important commodity in the livestock industry and is the number one red meat source consumed worldwide (Rothschild, 2004). It is also important to North Carolina's economy. The North Carolina swine industry increased dramatically in the 1980's. North Carolina now produces 1.7 billion kg of pork. It is a major farm commodity adding about $1.5 billion to the economy each year. North Carolina ranks second among states and is responsible for 16.2% of US swine produced (NC Dept. of Agri., 2005). There is an increased demand worldwide for animal products due to higher incomes and increased population, thus requiring production of more pigs (Soller, 1998). Successful production requires that pigs grow rapidly and efficiently, are disease resistant, have high carcass and meat quality, have increased survivability, and have high reproductive success (Rothschild, 2004).

Reproductive traits are an important component of improving efficiency of swine production. Litter size is an economically important reproductive trait and has potential to greatly enhance economic returns if increased (Haley et al., 1988). Litter size is lowly heritable (h^2=0.1), sex-limited, and not measurable until sexual maturity, making it difficult for producers to increase litter size using traditional selection methods. However, enough genetic variation does exist to improve litter size.

Traditionally, selection decisions for reproductive traits were based on phenotypic measurement and calculation of breeding values. Significant genetic improvement has been realized using breeding values and selection indexes. However, this method has limitations including difficulty of determining which animals carry the most favorable alleles (Dekkers and Hospital, 2002).

Advances in molecular genetics and limited successes in selecting for improved reproductive performance have encouraged scientists to look for single genes that may affect fecundity (Rothschild, 1996). Improvement of genetic maps has increased the number of markers available to do association studies. Polymorphisms can be used as markers to detect quantitative trait loci (QTL; Davis and Denise, 1998). Measurements of genetic variation can be studied at the DNA level, and effect of alleles on phenotype can be analyzed (Beuzen et al., 2000). Biological and physiological causes of genetic variation can then be determined with an understanding of sources of phenotypic variation at the DNA level (Haley and Archibald, 1998). Markers that are linked to phenotypic expression of the trait can be used through marker-assisted selection to select animals that are carrying the most favorable alleles. Genetic tests for validated candidate genes could be used by breeders to select animals for the next generation.

A possible candidate gene for litter size is follistatin. Follistatin is a monomeric glycoprotein encoded by a single gene that controls expression of follicle stimulating hormone (FSH) through its binding to activin. Presence or absence of follistatin helps determine whether animals enter into a reproductive phase (Patel, 1998). It has been shown that follistatin is essential for life. Follistatin deficient mice were smaller than their littermates, had multiple defects, and died shortly after birth (Matzuk, 1995b). Recent studies show mice lacking the follistatin gene in their granulosa cells, had a reduction in litter size and in number of litters per month (Jorgez et al., 2004). Through its effect on reproduction and development, follistatin is likely a candidate gene for litter size.

To date, despite advances in molecular technology, molecular genetics has made little direct contribution to animal breeding (Beuzen et al., 2000). Scientists hope to see this trend

change with use of marker-assisted selection. In the future, information from DNA sequences or genetic markers associated with improved production traits will likely be supplemented with traditional phenotypic selection methods when making selection decisions (Parmentier et al., 2001). The objective of this research is to determine if follistatin is associated with increased litter size.

Materials and Methods

Population Establishment

Animals from a Large White-Landrace composite population at Tidewater Research station in Plymouth, NC were randomly placed within litter to either a control line (Line C) or a select line (Line S). In Line S, direct selection for increased litter size, based on breeding values for number of pigs born alive (BA), was practiced for twelve generations. Selection in the contemporary control line aimed to keep estimated breeding values near zero (Holl et al., 2003, Blowe, 2004). Maternal effects of litter size were decreased by standardizing litters within 24 h of birth so that gilts were reared in litters with fewer than ten pigs. Measurements were recorded for number of fully formed pigs (FF), number of pigs born alive, number of mummified fetuses (MUM), and number of stillborns (SB).

Sample Collection

Blood samples were collected from animals in generation ten, born in 2000, using vacutainers containing Tris EDTA. Blood samples were transferred to microcentrifuge tubes, labeled with animal id, and stored at -20°C. Tail tissue was collected from each animal in generation eleven, placed in containers, labeled with animal id, and stored at -20°C (Blowe, 2004). Hair samples from animals in generation twelve were collected and placed in

bags labeled with litter and animal number as well as the date collected. Samples were stored at room temperature.

DNA Extraction

Samples of DNA from 3 ml of blood were extracted using Gentra's Puregene® DNA Purification Kit. First, red blood cells (RBC) were lysed followed by lysis of nucleated white blood cells (WBC). Protein was precipitated into a pellet form, and supernatant containing DNA was transferred to a new microcentrifuge tube. Following ethanol precipitation, samples were hydrated in a Tris EDTA solution. The Puregene® DNA Purification Kit for mouse-tail tissue was implemented in extracting DNA from pig tails by substituting porcine tissue into the protocol. Hair samples were extracted using the following procedure. Hair follicles (8-12) were placed into 200µl lysis buffer (500mM KCl, 100mM Tris-HCl at pH 8.0, 0.1µg/ml gelatin, 0.45% Triton X-100, 0.45% Tween-20, 0.5 mg/ml proteinase K) and digested at 55°C for four hours. Samples were then centrifuged at 5000g for two minutes and the clear aqueous layer was transferred to a new tube. Then 1.25µL of 4mg/ml RNase A was added to each sample. Samples were extracted with phenol/chloroform and ethanol precipitated. Next, DNA was resuspended in 50µL of 10mM Tris-HCl at pH 8.0 (Schnabel et al., 2000). Finally, DNA from all three generations was placed in a 384 well plate and stored at 4°C.

PCR

Follistatin is mapped to position 36.5cM on SSC16. Markers selected were predicted to map near follistatin, have high heterozygosity and a large number of alleles, and lacked known null alleles. Follistatin I, a type I marker, was used along with four type II markers including CGT27 (33.2cM), S0298 (33.2cM), S0363 (37.4 cM), and SW1661 (29cM). The forward

primers of the type II markers were labeled with different fluorescent dyes to maximize capillary efficiency. Marker information can be found in Table 1. Each 12µL PCR reaction contained genomic DNA (20ηg/µL), 1.3x buffer (Promega, no Mg^{++}; Madison, WI), 1.3x PCR enhancer (Eppicentre; Madison, WI), 2mM $MgCl_2$, 0.2mM of each dNTP, 0.2µM fluorescently tagged forward primer, 0.2µM reverse primer (Sigma Genosys; The Woodlands, TX), and 0.3 units *Taq* polymerase (Promega). Temperature gradients were performed with temperatures ranging from 50°-65° C to determine optimum annealing temperatures. Each reaction was visualized on a 1.5% agarose gel to determine each primer's optimal annealing temperature. Annealing temperatures for each marker are listed in Table 1. Problems with amplification of DNA existed for marker SW1661. Magnesium levels were increased from 2mM to 3mM for better amplification results. PCR reactions were run on the Eppendorf Mastercycler ep384 (Westbury, NY) for all 363 animals, and DNA was amplified for all five markers. PCR conditions were as follows: 94°C for five min followed by 30 cycles of 94°C for 30 sec, 30 sec at the appropriate annealing temperature, 72°C for 30 sec, followed by a final extension step at 60°C for 20 min.

Restriction Digest

Samples from all three generations amplified with the type I follistatin marker were digested with a restriction endonuclease. PCR product was added to master mix consisting of 1x buffer and 1 unit restriction enzyme (MspI; Promega) in a total volume of 10 µL. Samples were digested at 37°C for 1.5 hours and then held at 4°C. Samples were visualized on a 2.5% agarose gel and genotypes were manually scored. Samples that contained a band of 220bp were designated as AA and those with a band of 425bp were designated BB. Samples that contained both bands were designated AB. Genotypes are depicted in Figure 1. Genotypes

from generations ten and eleven were compared to those done by Blowe (2004) and discrepancies were resolved.

Genotyping

Genotyping animals for microsatellite markers CGT27, S0363, S0298, and SW1661 was done by using automated fragment analysis. Pigs genotyped were from either a line selected for increased number born alive (BA) or a contemporary control line. Amplified PCR products for each animal and marker were diluted as shown in Table 1. Diluted PCR product was coloaded in the ABI3100 capillary array by combining markers with different fluorescent labels or different allele size ranges. Samples were prepared for DNA fragment analysis by adding formamide and GSHD400-ROX size standard and processed using the ABI 3100 Genetic Analyzer from Applied Biosystems. Animals were genotyped using GeneMapper® software version 3.0, which compared fluorescent dyes to the size standard and assigned decimal values for each peak.

Data Analysis

Marker allele differences for four traits including number of fully formed piglets (FF), number born alive (BA), number of stillborn piglets (SB), and number of mummified fetuses (MUM) were tested in both Line C and S. A mixed-model analysis was used as suggested by Kennedy and colleagues (1992) to produce unbiased estimates of additive and dominance effects and to account for the effects of polygenes. The MTDFREML program (Boldman et al., 1993) was used to analyze the data using the full animal model. Pedigree relationships were taken into account. Fixed effects were year and marker genotype. Contrasts between genotypes were performed to estimate additive and dominance effects, and t-values were calculated to determine significance by dividing the difference between

genotypes by the standard error. Breeding values and inbreeding coefficients for each trait and generation were calculated by analyzing each trait with the fixed effect of year using MTDFREML and using PROC MEANS (SAS Inst., Cary, NC) to get averages by line and generation. Allele frequencies were calculated for all markers by generation for all animals and in control and select lines by dividing the total count of an allele by twice the number of observations in that line. A chi square analysis was done to test if the population was in Hardy-Weinberg Equilibrium. The formula $(O-E)^2/E$ was used, where O is the observed number of alleles, and E is the expected number of alleles. Degrees of freedom for each marker were calculated by subtracting number of alleles from number of genotypes. A continuity correction was performed to account for genotypes with fewer than five observations ($(|O-E|-0.5)^2/E$ (Yates, 1934). Variances for differences between allele frequencies in the control and select lines that did and did not account for random drift were also calculated. The formula $V_{ps-pc} = p_{gc}q_{gc}(1/[2N_{gc}]+F_{gc}) + p_{gs}q_{gs}(1/[2N_{gs}]+F_{gs})$ from Linville et al. (2001) was used to account for random drift, where p and q are allele frequencies, c and s represent control and select lines, respectively, g is generation, N is number of individuals with genotypic data per line, and F is average inbreeding. The variance for differences in allele frequencies that did not account for random drift was calculated using the formula $V_{ps-pc} = [(p_{gs}q_{gs})/(2N_{gs})]+[(p_{gc}q_{gc})/(2N_{gc})]$. Standard errors were calculated by taking the square root of each variance.

Results

Estimated breeding values and inbreeding coefficients are presented in Table 2. As intended, average breeding values in Line C are close to zero in all generations. Line S shows increases in mean breeding values for all three generations for FF, MUM, and SB. Mean

breeding value for BA increased in generation 11 but showed a slight decrease in generation 12.

Allele frequencies for follistatin, CGT27, S0363, S0298, and SW1661 are shown overall and separated by line in Table 3. Follistatin and S0363 had two alleles, S0298 had three alleles, and CGT27 and SW1661 had four alleles. Blowe (2004) previously noted that the B allele was the favorable allele for follistatin. However, effects of genotype were not significant, and therefore, favorable alleles cannot be determined in this study.

Lines within generation were tested for Hardy-Weinberg Equilibrium using a chi square test. Tables 4 and 5 show chi square values by line and generation for follistatin and S0363, respectively. Results are not shown for markers CGT27, S0298, and SW1661 because observed sample sizes for genotypes were small. Markers were not expected to be in Hardy-Weinberg proportions because assumptions for Hardy-Weinberg equilibrium were violated. The population is small and underwent selection for twelve generations. Values that were greater than the listed chi square value were considered significant, meaning they were not in Hardy-Weinberg equilibrium. In all three generations, the population was consistent with Hardy-Weinberg equilibrium for both the follistatin and S0363 marker.

Differences between allele frequencies by generation are listed in Table 6. Differences between select and control lines were different from zero. When drift was taken into account, standard errors were increased, and differences were non-significant.

Additive and dominance effects for follistatin and S0363 are listed in Table 7. Tables were not included for markers CGT27, S0298, and SW1661 because all possible genotypes were not represented in the population. Significance was determined based on calculated t-

values. Contrasts between genotypes for follistatin and S0363 in all traits showed no significant differences.

Discussion

Blowe (2004) chose follistatin as a candidate gene for litter size in pigs based on its biological function in reproduction and effects seen by knocking out the gene in mice. Research has shown that hypothalamic, pituitary, and gonadal hormones usually play a role in reproductive traits (Igarashi et al., 1993). Increased proliferation has been associated with growth and development of primordial follicles up to the ovulatory stage (Knight and Glister, 2001a). There has also been evidence that locally produced growth factors act as co-regulators of folliculogenesis (Knight and Glister, 2001b). Some growth factors that are thought to be associated with reproduction are inhibin, activin, and follistatin. Activins and inhibins are both part of the TGF-β superfamily of proteins and are structurally similar (Phillips, 2005). Follistatin is structurally unrelated to activin and inhibin, but is functionally linked through its binding ability (Knight and Glister, 2001a; Phillips, 2005). Together, all three function to regulate production of follicle stimulating hormone (FSH).

Follistatin is encoded by a single gene and is a cysteine rich monomeric polypeptide that is composed of about 36 cysteine residues (Patel, 1998; Muttukrishna et al., 2004). The follistatin protein varies in size from 32-39kDa and the gene is mapped to SSC16 (Muttukrishna et al., 2004; Ellegren, 1993). It contains a region encoding for a short signal peptide and 5 other exons. It is subject to alternative splicing which normally takes place at the 3’ end of the gene and has the option of keeping exon 5. Follistatin is most abundant in the pituitary, gonads, and kidney, but can be found in any tissues where activin is present (Michel, 1993).

Inhibin is found in the pituitary and gonads and functions as a reproductive hormone to enhance theca cell production of luteinizing hormone (LH)-induced androgen. It may also be involved in production of progesterone by luteal cells (Knight and Glister, 2001b). It also inhibits production of FSH (Knight and Glister, 2001a).

Activin is also involved in the pituitary and gonads as well as a large variety of organs such as the CNS, bone marrow, and pancreas (Igarashi et al., 1993). Activin stimulates granulosa cell proliferation and differentiation and also promotes synthesis and release of FSH (Igarashi, 1993; Knight and Glister, 2001a; Muttukrishna, 2004; Gladney et al., 2004). It is also involved in folliculogenesis. Activin's ActRcII receptors are necessary for folliculogenesis to occur. If interaction with type II receptors is prevented by follistatin, folliculogenesis will not occur (Matzuk et al., 1995a; Shimonaka et al., 1991). Follistatin has a great affinity for binding activin, and to a much lesser extent, it binds inhibin. Because activin has two β-subunits and inhibin has one, two molecules of follistatin can bind to activin, while only one molecule can bind to inhibin (Shimonaka et al., 1991). Binding of both β-subunits causes follistatin to prevent activin's interaction with its receptors and thus modulates production of activin in granulosa cells (Cataldo et al., 1994). Absence of follistatin allows activin to promote synthesis of FSH while also inhibiting secretion of growth hormone (GH), prolactin (PRL), and adrenocorticotropic hormone (ACTH). Under these circumstances, the animal enters into a reproductive phase (Patel, 1998). High levels of follistatin cause it to bind to activin, which blocks interactions of activin with receptors on target cells and prevents activin from acting on other tissues (Knight and Glister, 2001b). Once follistatin binds to activin, levels of free activin decline. This binding causes inhibition of synthesis of FSH as well as release of GH, PRL, and ACTH. Therefore, the animal enters

a non-reproductive state (Patel, 1998). Follistatin also binds to inhibin, but with a much lower affinity. Binding of follistatin does not seem to decrease inhibin activity like it does activin (Knight and Glister, 2001b).

Studies done in mice show that follistatin is an essential protein for life. Matzuk and colleagues showed that mice died shortly after birth when the follistatin gene was knocked out (1995b). Mice that were deficient in follistatin also grew slower than those with follistatin, had shiny, tight skin, delayed incisor development, decreased muscle mass, skeletal abnormalities, were lacking a hard palate, and were smaller than their littermates. In 1998, Guo et al. produced transgenic mice that overexpressed follistatin. The transgene was expressed in a variety of tissues. Mice that were overexpressing follistatin developed normally, and no deleterious effects were discovered, however, reduced fertility was observed in these mice. Reduction in fertility correlated with amount of overexpression. Overexpression of follistatin resulted in males having smaller testes and females having thinner uteri, showing that follistatin is clearly important in sexual development.

In 2004, follistatin was shown to affect reproduction in mice that lacked follistatin in granulosa cells. Mice with the follistatin gene deleted in their granulosa cells showed a reduction in litter size and number of litters per month. Some mice were infertile. Histological examinations showed that number of follicles was drastically reduced by six months of age (Jorgez et al., 2004). These effects seen in mice and the biological role of follistatin make it a likely candidate gene for litter size in pigs.

Blowe previously studied effects of follistatin on litter size in generations 10 and 11 (2004). A polymorphic site was discovered in an intron within follistatin. Data were analyzed using PROC GLM (SAS Inst., Inc., Cary, NC) with fixed effects of farrowing season, line,

genotype, and genotype*line and dependent variables were breeding values for FF, BA, and MUM estimated with MTDFREML. Results showed the genotype*line interaction term to be significant for FF, BA, and MUM and genotypes were significant for FF and MUM. The LS means for each genotype by line showed that genotypes differed significantly from each other. However, the study performed by Blowe had a limited number of animals. Therefore, a third generation was added to the study.

Data in an association study are normally either analyzed using a general linear regression model or a mixed model. To date, many studies have used a general linear regression model. However, these types of analyses tend to ignore relationships among individuals due to polygenes. A correlation exists between data from related individuals (Haley and Archibald, 1998). When these relationships are ignored, this type of analysis can lead to an increase in false significant effects no matter if selection has or has not been practiced. Spurious results are caused by an underestimation of standard errors that are associated with genotypic effects and lead to an increased probability of a type I error (Kennedy et al., 1992; Allen-Brady et al., 2003). This is particularly true if data from several generations are used when looking for an association. Kennedy and colleagues (1992) found that when selection was practiced for at least five years, an incorrect association was seen between a gene and a trait 29% of the time. Use of an animal model, which accounts for all known relationships between individuals by using pedigree information, is the preferred method of analysis for an association study. Kennedy et al. (1992) suggest using genotype as a fixed effect in order to properly estimate standard errors. Therefore, this study analyzed data using an animal model with MTDFREML. Fixed effects included year and marker genotype. Contrasts between genotypes showed that additive and dominance effects were

non-significant for all markers. Therefore, follistatin does not significantly affect litter size in the population tested.

Implications

The effects of follistatin on litter size in a control line and a line selected for litter size over twelve generations were studied. The mean breeding value for FF was 0.72 pigs greater in the select line than the control line in generation 12. However, the markers studied did not explain the differences seen between the lines.

Table 1. Marker sequences, restriction enzymes, allele sizes, relative position on SSC16, annealing temperatures, and dilutions [a]

Marker	Primer Sequence	Restriction Enzyme	Allele Size (bp) A	B	C	D	Relative Position	Annealing Temperature (°C)	Dilution
Follistatin	GGACCGAGGAGGACGTAAAT GGCCTTTCCAGGTGATGTTA	MspI	220 225	425	----	----	36.5	54.5	---
CGT27	TTCTGTTTAGTGGGCCTGAG TCTCACTGAATGCCTTTGTAAG	N/A	142	146	148	150	33.2	58.0	1:50
S0363	TAACTTGGATGCTGATAGCAC CATGGTTAAAATGGTTAACTGC	N/A	183	199	----	----	37.4	58.0	1:25
S0298	ACATAACATCGTAAATCAGC CTCCATCACAGGTCTCACA	N/A	171	173	175	----	33.2	60.0	1:25
SW1661	CCTCTTGTGGTTTGATGACTATC TCAACAACTTAAAGCAATCTCATG	N/A	189	193	195	199	29	62.0	1:25

[a] Primer sequences are listed from 5'to 3'. CGT27, S0363, S0298, and SW1661 are type II markers and therefore, do not have restriction enzymes. Optimal PCR was achieved at listed annealing temperatures. Optimal genotyping was achieved with listed dilutions.

Table 2. Mean breeding values for litter traits and inbreeding coefficients by line and generation

Trait [a]	line [b]	Generation 10	Standard Deviation	Generation 11	Standard Deviation	Generation 12	Standard Deviation
FF	C	-0.020	0.3028	0.012	0.263	0.061	0.257
	S	0.636	0.402	0.748	0.278	0.787	0.251
BA	C	-0.059	0.229	-0.0280	0.188	-0.002	0.177
	S	0.599	0.353	0.691	0.244	0.687	0.192
MUM[d]	C	0.685	0.662	-0.633	0.962	-0.757	0.726
	S	1.059	0.993	1.188	1.109	1.678	1.292
SB[d]	C	0.209	4.249	-0.667	4.441	0.268	5.343
	S	1.101	6.123	2.035	6.714	5.025	6.121
Inbreeding	C	0.084	0.021	0.108	0.021	0.149	0.018
Coefficient [c]	S	0.134	0.024	0.159	0.037	0.180	0.035

[a] FF, BA, MUM, and SB represent number fully formed, number born alive, number mummies, and number of stillborn, respectively.
[b] C and S represent control and select lines, respectively.
[c] Average inbreeding was calculated using all individuals in each line per generation.
[d] $x10^{-7}$

Table 3. Allele frequencies overall and within control and select lines for each generation [a]

Marker	Allele	Overall	Control	Select
Follistatin (10)	A	0.495	0.666	0.418
(n=97)	B	0.505	0.333	0.582
Follistatin (11)	A	0.504	0.674	0.419
(n=139)	B	0.496	0.326	0.581
Follistatin (12)	A	0.485	0.778	0.323
(n=101)	B	0.515	0.222	0.677
CGT27 (10)	A	0.540	0.196	0.7
(n=88)	B	0.063	0.143	0.025
	C	0.190	0.25	0.158
	D	0.210	0.411	0.117
CGT27 (11)	A	0.475	0.179	0.620
(n=118)	B	0.064	0.154	0.019
	C	0.292	0.244	0.316
	D	0.169	0.423	0.044
CGT27 (12)	A	0.454	0.153	0.629
(n=98)	B	0.046	0.125	0
	C	0.306	0.236	0.347
	D	0.194	0.486	0.024
S0363 (10)	A	0.194	0.214	0.185
(n=93)	B	0.806	0.786	0.815
S0363 (11)	A	0.190	0.206	0.187
(n=137)	B	0.81	0.804	0.813
S0363 (12)	A	0.271	0.232	0.291
(n=120)	B	0.730	0.768	0.709
S0298 (10)	A	0.878	0.875	0.879
(n=98)	B	0.087	0.016	0.121
	C	0.036	0.109	0
S0298 (11)	A	0.793	0.862	0.756
(n=135)	B	0.159	0	0.244
	C	0.048	0.138	0
S0298 (12)	A	0.815	0.878	0.782
(n=119)	B	0.143	0	0.218
	C	0.042	0.122	0
SW1661 (10)	A	0.026	0.052	0.015
(n=95)	B	0.037	0	0.053
	C	0.047	0.017	0.061
	D	0.889	0.931	0.871
SW1661 (11)	A	0.035	0.068	0.017
(n=130)	B	0.019	0	0.029
	C	0.046	0	0.070
	D	0.900	0.932	0.884
SW1661 (12)	A	0.021	0.058	0
(n=121)	B	0.008	0	0.013
	C	0.041	0	0.064
	D	0.930	0.942	0.923

[a] Overall allele frequencies include all animals genotyped for generations 10, 11, and 12. Number (n) of animals genotyped per generation is listed. Control and select lines are from white lines at North Carolina State University. The control line was maintained by selecting to keep the estimated mean breeding value at zero. The select line was selected for increased litter size for twelve generations.

Table 4. Observed numbers of genotypes for follistatin by generation in both lines and calculated chi square values for Hardy-Weinberg Equilibrium

Marker	Genotype	Control	χ^2	Select	χ^2
Follistatin	AA	12		10	
(10)	AB	16	0.613	36	0.445
	BB	2		21	
Follistatin	AA	20		15	
(11)	AB	22	0.120	48	0.176
	BB	4		30	
Follistatin	AA	21		4	
(12)	AB	14	0.136	34	1.848
	BB	1		27	

Observed numbers of genotypes for each marker are listed for control and select lines. A continuity correction was made due to small sample sizes. All three generations were in Hardy-Weinberg equilibrium.

Table 5. Observed numbers of genotypes for S0363 by generation in both lines and calculated chi square values for Hardy-Weinberg Equilibrium

Marker	Genotype	Control	χ^2	Select	χ^2
S0363	AA	0		1	
(10)	AB	12	0.971	22	0.433
	BB	16		42	
S0363	AA	1		0	
(11)	AB	16	0.113	34	3.612
	BB	29		57	
S0363	AA	0		3	
(12)	AB	19	2.478	40	3.240
	BB	22		36	

Observed numbers of genotypes for each marker are listed for control and select lines. A continuity correction was made due to small sample sizes. All three generations were in Hardy-Weinberg equilibrium.

Table 6. Differences in allele frequencies for follistatin between lines S and C

Generation	S-C	SE w/drift	SE w/o drift
10	0.249	0.238	0.074
11	0.255	0.258	0.061
12	0.455	0.263	0.064

Selection for FF was practiced for twelve generations in select line, and control line was selected to keep the estimated breeding values around zero.

Table 7. Contrast of marker genotypes [a]

Marker	Effect [b]	Line [c]	FF	SE	BA	SE	MUM	SE	SB	SE
Follistatin	a	c	1.651	1.188	1.353	1.152	0.178	0.120	0.395	0.375
	d	c	-0.770	0.710	-0.256	0.698	0.047	0.074	-0.273	0.227
	a	s	-0.117	0.726	-0.085	0.716	0.037	0.063	-0.056	0.218
	d	s	0.736	0.468	0.773	0.464	-0.035	0.041	-0.043	0.141
S0363	a	c	0.553	2.647	0.970	2.597	-0.130	0.271	-0.236	0.852
	d	c	0.780	1.413	0.810	1.387	-0.050	0.144	3.295*	0.451
	a	s	-0.564	1.496	-0.832	1.482	-0.128	0.129	0.286	0.450
	d	s	-0.063	0.813	-0.179	0.806	0.001	0.071	0.121	0.245

[a] Contrast between marker genotypes for follistatin 1 and S0363. Traits analyzed include number born alive (BA), number fully formed (FF), number still born (SB), and number mummified fetuses (MUM).

[b] 2x additive (a) and dominance (d) effects were estimated with MTDFREML.

[c] Lines were select (s) and control (c). The select line was selected for increased FF for twelve generations.

* $x10^4$

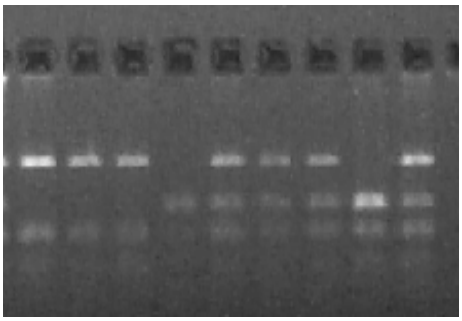

Figure 1. Gel image of follistatin 1 polymorphisms. The first three lanes are BB individuals. Lane 4 is an AA individual and lanes five, six, and seven are AB individuals.

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APPENDIX

GeneMapper® 3.7 Instructions

Compiled by Keri Boyette, Sarah Muncie, and Audrey O'Nan

A few "GeneMapper®" terms to become familiar with before starting:
Panel Manager-Where you create and set up all your marker information.
Bin Set-The individual marker used. You will want to name this the same thing you named the analysis method.
Kit-The overall name for your project (ex. Chromosme 18 Cattle Genotyping Project)
Panel-The markers you used for that particular project. Just so you know, you can use more than one marker when genotyping animals as long as they have a different allele size OR dye color. This is called multiplexing.
Autobin-when the computer looks at your data and selects where the alleles should be based on the allele information you set up in the Panel Manager.

STEP ONE-ADDING SAMPLES TO GENEMAPPER®

1. Open GeneMapper® 3.7 software.
2. Click FILE and ADD SAMPLES TO PROJECT.
3. Highlight the appropriate folder. At the bottom of the window, click ADD TO LIST. Your sample folders should appear on the right half of the window.
4. Click ADD (on bottom right side of screen).

STEP TWO-SETTING UP ANALYSIS PARAMETERS

1. On the toolbar, click TOOLS and PANEL MANAGER.
2. Click on PANEL MANAGER in the left window so that it is highlighted in blue.
3. Go to FILE and NEW KIT. Name the KIT the overall name of your project. Leave the kit type as Microsatellite and click OK.
   WRITE DOWN KIT NAME_____.
4. Highlight the **Kit** you just made by clicking on it. It should be highlighted in blue when you click on it.
5. Next, you are going to create a PANEL. A PANEL is what marker(s) you ran on that specific plate. You add new PANELS by going to FILE and NEW PANEL. Note to multiplexers: You will name your panel the name of the two markers you used-EXA. BMS2078_BMS360
   WRITE DOWN PANEL NAME_____.
6. In the left window, click on the PANEL you just made. This will highlight it in blue. Go to FILE and NEW MARKER. Add each individual marker you used for the PANEL you made.
   WRITE DOWN MARKER NAME:_____.
7. Enter the MARKER information-You need to enter information only for the Marker Name, Dye Color, and Minimum and Maximum allele size. That is all you need to worry about here. *If you are entering more than one marker, repeat step 6.*
8. Go to BINS (on top toolbar).
9. Highlight in the left window the kit you created in step 3. (Remember, the kit pertains to the overall project name).

10. Go to BINS and NEW BIN SET.
11. Name the BIN SET the same thing you named the PANEL in step 5. I know that this is redundant but just go with it.
    WRITE DOWN BIN SET NAME:_____.
12. Click APPLY and OK. This should take you back to the main SAMPLES Screen.

STEP THREE-SETTING UP ANALYSIS METHOD

1. Locate the ANALYSIS METHOD column and click on the first row. A pull down screen will appear. Select NEW ANALYSIS METHOD.
2. Under SELECT ANALYSIS TYPE click microsatellite and then OK.
3. A window will pop up under the "General" tab. Name the Method something relevant to the plate you are analyzing. We commonly use the Marker Name. *Note to Multiplexers: You would enter both marker names (EXA. BMS2078_BMS360).* That is all you need to change in this window.
   WRITE DOWN ANALYSIS METHOD NAME:_____.
4. Go to the "Allele" tab. Click on BINSET and you will see a pull down menu. Select the Bin Set for you sample. This will have the same name as the Analysis Method. That is all you need to do on this screen.

STEP FOUR-FILLING IN SAMPLE INFORMATION

1. In the Main SAMPLES screen, click on the column named "Size Standard." A pull down menu should appear. Select GS400HD. Fill down the column by clicking the column header and holding down CTRL-D.
2. Click the "Analysis Method" column. Choose what ever you named the Analysis Method in the Step Three of the Step Three Section.
3. Click the "Panel" column. Click on the first cell and a pull down menu should appear. Click on the KIT folder and double click the PANEL name. Fill down the column by clicking the column header and holding down CTRL-D.

STEP FIVE-SAMPLE ANALYSIS AND AUTOBINNING

1. Click on the Green Arrow on the action toolbar.
2. Name the PROJECT (*you can name this the same thing you named the KIT)*
   WRITE DOWN PROJECT NAME:_____.
3. Wait for the data to be analyzed.
4. Now you will need to AUTOBIN. Don't fret, you are almost done!!!!! To autobin, go to TOOLS and PANEL MANAGER. Click on KIT> PANEL>BINS>ADD REFERENCE DATA. Select a couple of runs to use a reference data. Click ADD (bottom right side of window).
5. Go back to main SAMPLES screen and Click on the Green Arrow on the action toolbar (this too is redundant).
6. After it has reanalyzed your sampled, you can click on the GENOTYPES tab and see your analyzed data.