Genetic studies of Incubation behaviour and Morphological traits in Chickens

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Preface

The work contained within this thesis is my own and has not been done in collaboration, except where otherwise stated. The text does not exceed 70,000 words. No part of this thesis has been submitted to any other university in application for a higher degree.

Atta Basheer

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Abstract

Finding the genes that underlie variation in production and developmental traits has important economic applications. Incubation behaviour represents a loss of production in conventional breeds of chicken adapted to local conditions and was what motivated this thesis. The Mendelian traits of comb type, crest, Silkie and normal feathers, feathered leg, fibromelanosis, comb colour, skin and shank colour, feather colour and patterns are of interest because of the insight they give to genes and development and were also investigated in the thesis.

We used White Leghorn and Silkie lines of chicken to detect the genetic loci controlling incubation behaviour and Mendelian traits using linkage based analysis in an F2 cross. The evidence for QTL affecting incubation status over the whole period on chromosome 5 was strong ($P<0.05$). After the addition of 218 new informative SNP markers across the genome including chromosome 5 the 95% confidence interval spanned a region around 45 cM having previously been 95 cM. Three other suggestive QTL for incubation status were found after the addition of SNP markers on chromosome 1, 18, 19, E22C19W28 at 70, 0, 1 and 13cM respectively. The mode of action of the incubation status QTL indicates that the White Leghorn allele was either promoting incubation behaviour or that heterozygotes have performance that exceeds the homozygotes except the QTL on chromosome 1 where the Silkie allele is promoting incubation behaviour as might be expected. A highly significant QTL ($P<0.01$) for early incubation behaviour (25-30 weeks) was found on chromosome 8 at 18 cM. This QTL has an additive effect with the possession of a Silkie allele increasing the likelihood of incubation behaviour. Other suggestive QTL for early incubation behaviour were found on chromosome 26 and 1 at 0 and 66cM respectively.

For Mendelian traits, genome wide significant (P<0.01) genetic loci for comb type, crest type and feather type was found on chromosome 7 at 77cM, linkage group E22C19W28 at 7cM and on chromosome 3 at 169cM respectively. Significant genetic loci (P<0.01) for leg colour and skin colour were found on chromosome 20 at 56cM and 60cM respectively. In the present study, loci for all feather patterns were found on E22C19W28 even after removing animals carrying the dominant white alleles, suggesting dominant white or another allele at the locus was still influential.

Comb type and incubation behaviour were investigated at the gene level. Thyroid stimulating hormone receptor (TSHR) is believed to be involved in the process of domestication and was found at the peak position of the most significant QTL on chromosome 5 for incubation behaviour. Functional exploration of Wnt genes as a candidate gene for comb type was investigated by *in-situ* hybridization in Silkie and White Leghorn embryos. The Wnt6 gene showed expression in the region of the presumptive comb development of embryos.

In conclusion, for the first time genetic loci that explain maternal behaviour have been described. The coincidence of the incubation behaviour locus on chromosome 5 with the site of the strongest selective sweep in poultry, the TSHR, and the coincidence of QTL on chromosome 1 and 8 with thyroid hormone activity it would appear that the thyrotrophic axis may be critical to the loss of incubation behaviour and improved reproductive performance with domestication. Further analysis of these loci should be able to produce markers that can reduce the propensity for birds to incubate. Comb type marker might allow introgression of this trait to prevent comb damage in commercial hens.

Chapter 1 General Introduction

1.1 Genetic variation

Genetic variation is the naturally occurring genetic differences among individuals of the same species. This variation permits flexibility and survival of a population in changing environmental circumstances. Consequently, genetic variation is often considered an advantage for a species, as it is a form of preparation for the unexpected (Sawai et al., 2010).

Major forces creating genetic differences between breeds and populations are mutation and recombination, along with genetic drift, natural and artificial selection, and migration (Allendorf, 1986). During domestication, genetic differences between breeds and populations are mostly accumulated by isolating them from others and selecting for favourable traits (Andersson, 2001). Weight and Romanov (2001) categorized chicken genetic resources into the following five categories (1) wild populations, (2) indigenous breeds which comprise domesticated but unselected populations, (3) standardised breeds selected for morphological traits (mostly by fanciers), (4) chicken lines selected for quantitative traits, encompassing commercial layers and broilers and middle level dual purpose breeds, and (5) experimental populations and inbred lines. Others have proposed slightly different but generally similar classifications in the chicken (Crawford, 1990).

The potentially important genetic resources are those which are characterised by unique genetic features, on average, most distant within the species. Estimates of the degree of genetic uniqueness are usually produced through measurements of

between- population distances based on some genetic marker systems (Teale et al., 1994).

An immense amount of variation have been accumulated and observed in chicken breeds of the world due to thousands of years of selection (Jensen, 2006). This variation is due to artificial and natural selection acting on genetic variability in many genes during domestication and can be utilised to understand basic biology and gene function (Al-Nasser et al., 2007). Domestication has also led to genetic changes that affect quantitative traits in chicken. Sandercock et al. (2009) quantified the genetic variations for commercial yield traits, live weight, carcase yield, muscle quality and support organs in 37 lines of commercial broiler, layer and traditional chickens.

Advances in technologies have made it convenient to study the inheritance of many biological and commercially important traits in a more precise way and to detect QTL and genes which underlie quantitative traits (Kearsey, 1998). For a given trait, there are likely to be several or many genes segregating in a Mendelian fashion in any given population and their effects will be approximately additive (Kearsey, 1998). Quantitative traits are the traits which show continuous variation in a population and are more or less normally distributed. There is no obvious discontinuities in distribution as might be expected in classical, single gene trait, such as 1:2:1 distribution of genotype and phenotype in experimental crosses. Such single gene, qualitative genes, have a large effect on the phenotype compared to environment and, dominance apart, the genotypes have a recognizably different

phenotype. Very often one of the alleles is non-functional or very dysfunctional, which results in a clear phenotype (Kearsey 1998).

The genetic basis of quantitative variation, therefore, needs to take account of the properties of the genes individually- their gene frequencies and the magnitude of their effects on the trait of interest. These genes cannot be studies individually using the methods of classical Mendelian genetics because their effects are lost in the statistical 'fog' of all other background variations (Falconer and Mackay, 1996). Therefore specific methodologies were developed to try and locate the regions of the chromosome responsible for quantitative variation.

1.2 QTL detection approaches

QTL mapping is an important first step in the process to increase the understanding of the genetic basis of quantitative traits. The information about the co-segregation between traits and marker loci can be used for marker assisted selection (MAS), but also for characterisation of the genes influencing the trait (Broman, 2001).

The combination of molecular marker and trait data to explore the individual genes concerned with quantitative traits, QTL analysis, has become an important tool to allow biologists to dissect the genetics of complex characteristics (Kearsey, 1998). The use of markers to detect individual loci responsible for quantitative genetic variation (quantitative trait loci or QTL) provides much greater power than segregation analysis without marker information (Haley and Knott, 1992).

There has been a rapid increase in the development of QTL mapping methodologies since Lander & Botstein (1989) published their pioneering work on interval mapping

by using the maximum likelihood approach to map a QTL lying within a chromosome region surrounded by two markers (Ma et al., 2002). Likelihood ratio profile (or LOD) is obtained by calculating likelihood of a QTL across all intervals. Maximum-likelihood methods are, however, relatively complex and can be computationally slow.

Haley and Knott (1992) developed methods for mapping QTL based on multiple regressions which can be applied using any general statistical package. These regression methods produce very similar results to those obtained using maximum likelihood by using F2 population and are also used to explore the models with more than a single QTL. Other models, for example with more than two QTL, with environmental fixed effects, with between family variance or for threshold traits, could also be fitted by using this method. The regression method has become the method of choice for the analysis of QTL mapping data from inbred lines due to ease and speed of application and generality of regression methods for flanking marker analyses (Haley and Knott, 1992) and is now available in the form of web based application, Grid QTL (Seaton et al., 2006).

1.3 QTL experimental designs

The F_2, backcross (BC) and F_2 designs have been used in methodologies for QTL detection studies. Divergent populations are usually crossed to produce the first generation. One of the parental lines is then back-crossed to the F_1 in the second generation in the BC design, while in the F_2 design the F_1 are intercrossed and phenotypic information from the second generation is used for QTL mapping. A third generation may be produced from intercrossing the second generation in an F_2-F_3 design and the progeny in the third generation are assessed for QTL analysis. The F_2 is the most popular design used in chicken QTL studies where typically the F_2 cross between two lines produces hundreds of birds and is the approach used in the population studied for this thesis. Using interval mapping (IM) as proposed by Lander and Botstein (1989), Knott and Haley (1992) investigated the maximum-likelihood methods for QTL mapping in F2 populations using simulated data.

1.4 Traits of interest

Traits can be categorised as quantitative traits and qualitative traits. Quantitative traits are complex and are often economically important such as feed conversion ratio, growth rate or egg production (Hocking 2005), whilst qualitative traits are Mendelian e.g. feather colour and pattern, comb type, crest type, feather type, skin colour, feathered shanks (ptilopody) seen in the populations used in this study, but can include traits of commercial importance such as tainting of eggs (Honkatukia et al., 2005).

1.5 Quantitative trait

1.5.1 Incubation behavior

Incubation behaviour is a complex trait, resulting from interaction of the birds hormonal system and its environment (Sharp et al., 1997). The trait is characterised by persistent nesting, turning and retrieval of eggs, clucking and nest defence. It is associated with increased secretion of prolactin and decreased secretion of luteinising hormone and subsequent regression of ovaries and oviduct and cessation of egg production (Sharp, 2009, Romanov et al., 2002). The hormonal events and the environmental conditions, high temperature, darkness, presence of eggs and nest are key factors which encourage incubation behaviour (Hutt, 1949, Sharp, 1987). This behaviour is inhibited if eggs are removed from the nest, as they are laid and is one of the reasons for the use of battery cages in commercial poultry production.

The neural and hormonal basis of incubation behaviour has been studied extensively in some domestic species (Buntin, 1996) including the domestic fowl (*Gallus gallus*), turkeys (*Meleagris gallopavo*) (EL Halawani et al., 1985, EL Halawani et al., 1990, El Halawani et al., 1993) and domesticated ring doves (*Streptopelia risoria*) (Lea and Sharp, 1989, Lea et al., 1986) Most of the work is focused on hypothalamus-pituitary axis and the secretion of the hormone prolactin. The cause and effect relationship between incubation behaviour and prolactin can be studied more precisely in species in which continuous tactile input from the eggs is required to maintain elevated prolactin and broodiness (EL Halawani et al., 1980). In galliforms, their appears to be a constant reinforcement between the contact between the eggs and the brood patch and the behaviour manifest by an increase in prolactin secretion.

The development of a brood patch is one of the earliest morphological signs of incubation behaviour (Lea and Klandorf, 2002). Lea et al (1981) studied hormonal profiles and brood patch development in bantam fowl and found that an increase in brood patch score can be clearly seen after five days of incubation and was the first overt signs that hens were about to become commence incubation. Development of brood patch is stimulated by increased prolactin acting synergistically with plasma oestrogen (Lea and Klandorf, 2002). After the onset of incubation, when ovarian steroid secretion decreases, the brood patch is maintained by increased plasma prolactin.

Experiments were conducted to separate the influence of tactile and visual stimuli originating from the egg and nest on the development of incubation behaviour. During the laying period, eggs accumulate in the nest and females start to sit on the nest for longer and ultimately females incubate for almost the whole day and in the ovary follicle development becomes suppressed (Meijer, 1995). Visual and tactile stimuli inform the females about the number of egg and helps in the development of incubation behaviour (Michaela, 2001). Book et al. (1991) found that if eggs are removed continuously from the nest of turkeys, it shortened the time spent in the nest and also inhibited the full development of incubation behaviour in the turkeys

Incubation behaviour is associated with low concentrations of plasma luteinizing hormone (LH) and ovarian steroids, and with high and low concentrations of plasma prolactin, respectively. The laying of eggs is the pre-requisite for the initiation of incubation behaviour and in some cases birds showed signs of incubation behaviour after a number of clutches. Once a clutch of eggs is nearly to completion, tactile

information transmitted from the brood patch passes up the spinal cord to the hypothalamus to ultimately stimulate the secretion of vasoactive intestinal polypeptide (VIP) from basal hypothalamus. This neuropeptide acts directly on the avian anterior pituitary gland to release prolactin (EL Halawani et al., 1990, Macnamee et al., 1986) and these prolactin secretions transforms nesting into incubation behaviour (Sharp, 2009). Tactile signals from the brood patch also inhibit the synthesis of GnRH-I and may increase GnIH synthesis, which additionally suppresses gonadotrophin secretion (Sharp, 2009).

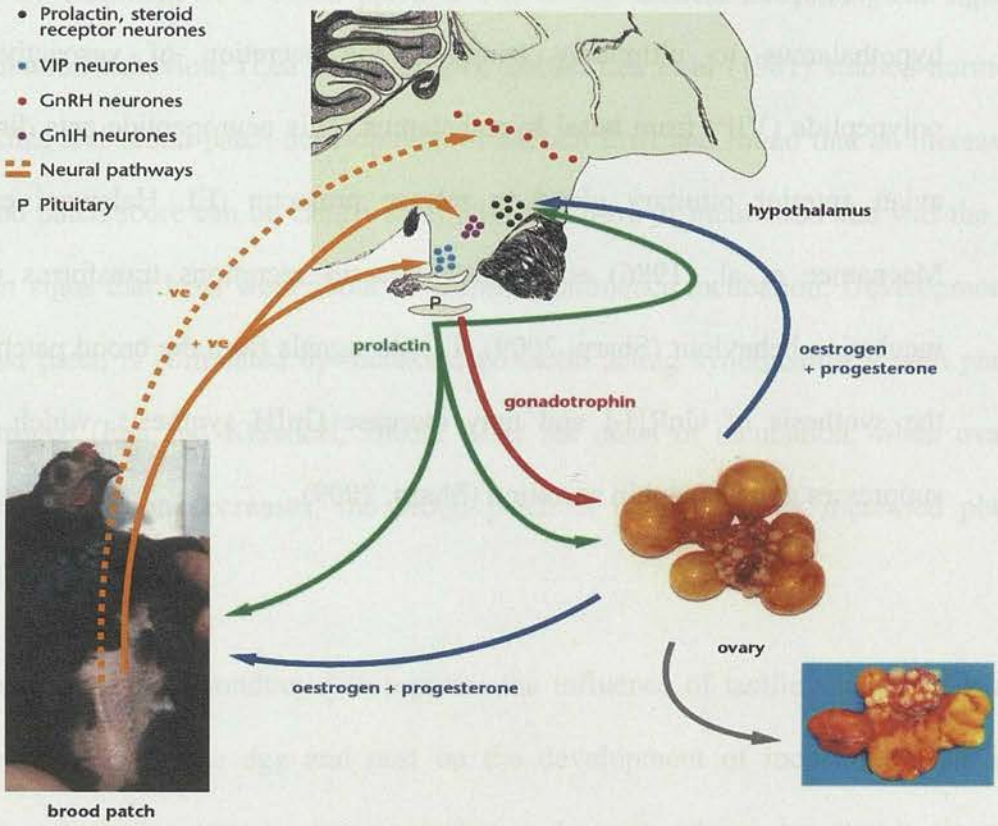


Figure 1-1 Neuroendocrine interactions in incubating domestic hens. Ovarian steroids stimulate brood patch development in hens about to become broody and prolactin subsequently maintains it. Tactile stimuli from eggs are transmitted via the brood patch to the hypothalamus to inhibit neurones containing gonadotropin releasing hormone-I (GnRH-I), and to stimulate neurones containing gonadotropin inhibitory hormone (GnIH) and the avian prolactin releasing hormone, vasoactive intestinal polypeptide (VIP). This results in decreased gonadotropin secretion and increased prolactin secretion that in turn leads to ovarian regression (insert) and expression of incubation behaviour (Sharp, 2004)

Under domestication and selection pressure incubation behaviour has been eliminated in certain breeds of chicken, notably the White Leghorn but still exists in many breeds of poultry including Red Jungle fowl (Collias and Collias, 1967). This is hardly surprising since all birds that incubate their own eggs must show this behaviour if they are to reproduce. The Egyptians, followed by the Chinese, perfected artificial incubation methods at least 3000 years ago (Banner, 1916). Eventually this lead to the revolutionary technological development of the electric incubator in the mid of 20th century that was a pre-requisite for the development of modern lines of chickens that do not display incubation behaviour and the breaking of the link between maternal behaviour and reproduction (Megyesi and Henson, 2011). Because incubation behaviour is associated with a cessation of reproduction for many weeks it is clearly desirable to remove this trait to produce highly productive egg laying strains. The original jungle fowl lays 10 to 15 eggs per year in the wild whilst modern strains manage 300 eggs per year, a feat impossible if they showed any symptoms of incubation behaviour (Moreng and Avens, 1985, Romanov and Weigend, 2001)

The White Leghorn (WL), a breed of the Mediterranean class has high egg production and low or no incidence of incubation behaviour (Hutt, 1949) and there has been a belief that a major gene may be responsible (Romanov et al., 2002, Romanov, 2001). On the other hand, the Silkie breed, bred mainly for ornamental purposes, carries alleles for many morphological traits including polydactyly (Po), silkie feathering (h), fibromelanosis (Fm) and rose comb (R) (Hutt, 1949) and in the context of this thesis it has a high incidence of incubation behaviour (Liang et al.,

2006). The Silkie breed belongs to the Asiatic class and is thought to have originated in China (van Wulfften Palthe, 1992).

Breed difference in the degree of incubation behaviour showed that this trait was likely to have a significant genetic component (Hutt, 1949). The genetics of incubation behaviour has been investigated and has produced conflicting observations. Punnet and Bailey (1918) showed that incubation behaviour is controlled by more than one independent autosomal gene. Later, it was reported that this trait is controlled by sex linked genes (Saeki, 1957, Saeki and Inoue., 1979). Whilst Saeki (1957) demonstrated that this traits is polygenic with a major sex linked contribution i.e. the chicken Z chromosome might contain a major gene (or genes) controlling the expression of incubation behaviour. Romanov et al. (2002) investigated the genetic control of incubation behaviour in domestic hens by analysis of behaviour in reciprocal crosses between White Leghorn and Bantam and the back cross of F1 males and White Leghorn females. The incidence of incubation behaviour in Bantam and White Leghorn hens was 78.6% and 0% respectively and in F1 back cross it was 5.8 %. These results suggested that incubation behaviour was not controlled by major genes on the Z chromosome(Romanov et al., 2002).

These observations are consistent with the view that incubation behaviour in chicken is not controlled by major genes on the Z chromosome. There must therefore be major autosomal genes contributing to the expression of the behaviour. If a gene for incubation behaviour exists on the Z chromosome it was concluded it is one of at least three genes including two dominant autosomal genes, one causing and other one inhibiting incubation behaviour, with probably equal influence (Romanov, 2001).

It was suggested that modern sophisticated molecular genetic techniques and a resource population will be useful in the identification of quantitative trait loci for the expression of incubation behaviour. It will further smooth the process of detection of functional genes and marker assisted selection against incubation behaviour (Romanov, 2001).

Incubation behaviour is a major problem in conventional breeds of chicken but it still a problem in some of the commercial broiler lines especially dwarf lines. In developing countries, traditional poultry make up a significant proportion of poultry in national flocks, a good example is the Desi chicken in Pakistan (Anjum et al., 2012). These Desi chicken are contributing 40-50 % share of the total eggs and meat produced in Pakistan (Bhatti, 2002). In Europe and North America, the existence of backyard or farmyard poultry is an example of tradition poultry although there number is decreasing (Kitalyi, 1997). These village poultry play an important role to improve food security and assist in poverty alleviation in rural populations. Despite the impact of traditional poultry in poverty alleviation, there has been a lack of research to improve the efficiency of traditional poultry production (Kitalyi, 1997). Inefficiency of village poultry and low productive performance is in part due to incubation behaviour (Bhatti and Sahota, 1996). There has been little improvement in the number of eggs produced in traditional poultry production for the last 40 years but on the other hand they are very resistant against diseases and provide potentially interesting DNA to be incorporated into commercial founder lines. The reasons of low production (incubation behaviour) of poultry have not been subject to successful genetic improvement and are little studied. In conclusion, tradition poultry

production is making a difference to poverty alleviation, but there is scope for even greater impacts of poultry to the rural economy (Scanes, 2007).

1.6 Morphological traits

In certain parts of the world, consumers are willing to pay higher prices for tasty meat and for the appearance of the birds (Fanatico and Born, 2002, Zhou, 2002). According to consumer demand, producers need to look at the bird appearance, and adapt breeding strategies accordingly. In southern China and France, yellow plumage, yellow skin and yellow shank has been considered as indicator for high quality of meat and good luck (Yang and Jiang, 2005, Zanetti et al., 2010). In the live market, consumers pay much more attention to colour of plumage, skin and shank as well as redness and size of comb. Black skin and shank are widely accepted as a nutritive and tonic food for the consumer (Yang & Jiang 2005).

In this study, a cross of White Leghorn (WLH) and Silkie (SLK) has been analysed. The following qualitative traits are segregating in the F2 population most of which have Mendelian characteristics, so it will be possible to map the loci for these traits using the same approach as used for the quantitative traits.

1.6.1 Comb type

Comb is really the distinguishing character of genus Gallus. Most domestic breeds have a single comb. It consists of single blade which runs the length of the head and is topped by a varying number of points (Crawford, 1990). The size of the single comb varies among breeds but it is consistent within breed and size was speculated to be under genetic control (Hutt, 1949) and this was clear from selection

experiments (Tufvesson et al., 1999). This type of comb is the major characteristics of breeds of Mediterranean class like White Leghorn (Roberts, 1997). Rose comb is a breed characteristic of more than fifteen recognized breeds including Silkie and is characterized by broad comb, flat on top, covered with small regular papillae and ending with a spike or leader at the rear (Crawford, 1990).

Bateson (1902) quoted by Hutt (1949) demonstrated that single comb is recessive (r) to rose comb (R) and rose comb is inherited as a completely dominant gene. Poor fertility is associated with R/R males (Etches et al., 1974). Fertility of R/r and r/r males and all three genotypes of females is perfectly normal (Crawford, 1990). Sperm cells of R/R males have some fertilizing capability but they cannot compete with r/r males sperm (Petitjean and Servouse, 1981).

Cochez (1955) and Ponsignon (1951a) found that reproductive performance of rose comb birds was poorer than that of single comb birds. Crawford and Smyth (1964a) conducted extensive studies of the reproductive efficiency of birds of three comb genotype (RR, Rr, rr) and found that low fertility is obtained from homozygous rose comb male (RR). Kirby et al(1993) also found association of homozygosity of rose comb allele in male domestic fowl with reduced spermatozoa metabolic rate and motility. Dorshorst et al. (2010) in their study of fibromelanosis found associations of rose comb with certain SNPs on chromosome 7.

Finding QTL and genes for comb type is also a major consideration in the current study. Markers for this trait may be of interest to commercial breeding companies because due to cannibalism birds injure each other especially on the comb and

wattles. So it is a concern of the poultry breeding companies to minimize the comb size to potentially enhance animal welfare.

1.6.2 Crest type

A Crest is a predominant feature of a number of breeds including Silkie. This results from some of the feathers being unusually long and erect rather than fitted closely to the head (Hutt, 1949). Its phenotypic expression varies widely and this may be due to the size and type of comb associated with it. The inheritance of crest has been studied in chicken, Hurst (1905) quoted by Crawford (1990) reported that this trait is inherited by an incompletely dominant gene. It is also reported that only one pair of genes was involved in this trait (Davenport 1906 quoted by Crawford 1990). Dunn and Jull (1927b) assigned the gene symbol Cr to crest. It is still not known whether the full crest and the various gradations to the small crest are caused by same gene. In the literature, it is also found that size of crest is also directly proportional to the extent of abnormalities in the cranium and in the brain (Hutt, 1949, Tegetmeier, 1856, Rehkämpera et al., 2002).

1.6.3 Feather type

Different kinds of feather are divided into two classes. Neossoptiles include the down and nestling feather of newly hatched chick. While teleoptiles, include adult feathers of all kinds including the feathers known as contour feathers that are outermost. The normal contour feather of fowl consists of the central quill, shaft and vane (Hutt, 1949). In Silkie birds, the contour feathers have a delicate shaft and unusually long barbs. These are usually bifurcated, sometimes twice or more. In this case, the irregular arrangement of barbules accounts for the absence of a flat web in the

feather and the resultant distinctive Silkie appearance of the Silkie breed. This character is caused by autosomal recessive gene, h. its recessive nature was proved by (Bateson and Punnett, 1905-1908).

Zhang et al (2009) studied feather inheritance of six indigenous chicken breeds including Silkie in F1 and F2 population and found that feather types were controlled by a single gene H(h) located on euchromosome. Dorshorst et al (2010) found association of Silkie feathering with a 15.7 Mb region on chromosome 3.

1.6.4 Feathered leg (Ptilopody)

Ptilopody is the term used to describe feathering on the shank and toes (collectively on the legs). Leg feathering present in the fowl is one of the most difficult test because of degree of variation in feathers present in this regions (Dunn and Jull, 1927b) which make the things harder to map. Feathered leg is the major characteristic of the Silkie fowl. In the context of the present study, this trait is poorly phenotyped. Punnet and Bailey (1918) studied the inheritance of feathered shank by using two distinct crosses, In one case Langshan (feathered leg) was crossed with Brown Leghorn (clean leg) and in other case Langshan was crossed with gold pencilled Hamburg. They found that feathered leg was due to one pair of dominant genes.

Somes (1992) using Langshan and Brahma, Cochin and Sultan crossed with White Leghorn, showed that the Langshan and Brahma each carried one pair of dominant genes, while the Cochin and Sultan carried two pair of dominant genes. A Brahma X Langshan F1 back crossed to the White Leghorn revealed that these two breed may

share the same single locus, while a Sultan X Langshan F1 back crossed to the White Leghorn suggested that one of the two loci causing leg feathering in the Sultan was shared by the Langshan.

From the above results, it is concluded that basically, three loci are involved in the types of leg feathering that are considered as breed characteristics. There are two dominant genes at separate loci which when present together produce heavy leg feathering of the Cochin and Sultan. One or the other of these loci by itself produces the weak leg feathering characteristic of Langshan.

Dorshort et al. (2010) studied feathered leg and vulture hock simultaneously and found a single genomic region on chromosome 13 (SNP rs14999343 at 15.6 Mb) was significantly associated with both of these trait. They further demonstrated that ptilopody showed a higher degree of association with rs14999343 as compared with vulture hock and has a stimulatory effect on the vulture hock phenotype so from their results it is unclear which trait is truly associated with this region.

1.6.5 Fibromelanosis

Fibromelanosis is characterized by extensive pigmentation of the dermis, particularly in association with loose connective tissue of the deep dermis, as well as of the parietal and visceral peritoneum, the periosteum and the pericondrium. In addition numerous melanocytes are found around blood vessels and nerves and in association with the connective tissue stroma of the visera (Crawford, 1990).

Bateson and Punnet (1911 quoted by Hutt 1949) discovered that in Brown Leghorn, this trait is inhibited by a sex linked gene. Dunn and Jull (1927) found a similar type

of inhibitor in White Leghorn and named it as melanin inhibitor (*Id),* closely linked with the Z chromosome. Stolle (1968 quoted by Crawford 1990) reported that fibromelanosis is due to sex linked *id* in concert with a dominant enhancer.

Earlier workers reported considerable variation in the degree of fibromelanotic pigmentation, suggesting that a number of other factors can modify its expression (Crawford, 1990).

Dorshorst et al. (2010) used Genome-wide single nucleotide polymorphism (SNP)-trait association analysis to detect genomic regions showing significant association with pigmentation genes in 2 chicken mapping populations designed to segregate independently for *Id* and *Fm*. They showed EDN3 expression is increased in the developing Silkie embryo

s during the time of melanoblast proliferation. They also found that the SNP showed the highest association with *Id* was located at 72.3 Mb on chromosome Z and 10.3–13.1 Mb on chromosome 20 showed the highest association with Fm. Le Bihan-Duval et al. (2011) identified the causal gene (or QTG) underlying a highly significant QTL controlling the variation of breast meat colour between high-growth (HG) and low-growth (LG) chicken lines. They found two fully-linked single nucleotide polymorphisms (SNP) within the proximal promoter of BCMO1 (beta-carotene 15, 15'-monooxygenase), a good functional candidate gene. It is a key enzyme involved in the conversion of beta-carotene into colourless retinal.

1.6.6 Skin colour

Birds skin is mainly made of two layers, a comparatively thin outer one called epidermis and thicker inner layer, dermis. Skin colour in fowl arise from the presence, either singly or in combination, two principle pigments, melanin and Xanthophill (Hutt, 1949). Mainly, skin colours are white (e.g. White Leghorn) and black (e.g. Silkie). Bateson (1902 quoted by Hutt 1949) used a cross of White Dorking X White Leghorn and found that white skin is dominant to yellow. Zhang et al. (2000) observed the sex-linkage inheritance of skin colour in some local breeds of chicken. The results showed that there was a pair of melanin genes, PP, on autosomes in Taihe Silky Fowl, an Id (inhibitor of dermal melanin) gene on the sex chromosome in Xianju Fowl, Xiaoshan Fowl and Beijing You Fowl respectively. The offspring produced by crossing of Taihe Silky Fowl (Male) with the fowl (Female) carrying Id alleles (such as Xianju Fowl, Xiaoshan Fowl and Beijing You Fowl) can allow autosexing by skin color, male chickens have yellow skin and female chickens have black skin.

Huang et al (2003) studied the inheritance of skin colour and shank colour in Sichuan Mountain Dark-bone Chickens (SMDC), they conducted a cross between SMDC and Lingnan yellow meat chicken (LYMC). The results indicate that the skin colour and shank colour of day old chicks are incompletely stable in the intercrossing groups, and they change with age, which indicates the expression of genes that controls the deposition of melanin changes with the age of chickens. In the population which are produced by intercrossing between SMDC (♂) and LYMC (♀), almost all darkly pigmented chicks are female at age of 10 weeks. They also showed that the content

of dermal melanin in the chicken seems to have a relation with the mortality rate. Eriksson et al. (2008) found that yellow skin is caused by one or more cis-acting and tissue specific regulatory mutations that inhibit the expression of BCDO2 (beta carotene dioxygenase 2) in skin. They also found that yellow skin is originated from grey jungle fowl (Gallus sonneratii) rather than red jungle fowl (Gallus gallus).

1.6.7 Feather colour and Pattern

Feather colour is classified into two major classes, in one of these, the colour of the feather depends upon the presence and size and shape of the pigment. In the other case, structural colour not only depends upon the pigment present but also depends upon the way light is reflected, diffracted, dispersed and absorbed. Melanin is responsible for black colour and its variant forms give rise to light brown to brown colour of feather (Hutt, 1949). The symbol generally used to designate the presence of black pigment of any kind is C (colour) but birds with solid black pigment must also carry an allele, E, which permits extension of colour to all parts of the plumage. White colour feather is present in White Leghorn. Kerje et al. (2004) reported that at Dominant white locus, there are alleles of Dominant white, Dun, and Smoky which are affecting plumage colour in the domestic chicken. They used red jungle fowl/White Leghorn intercross and did not find any recombination in linkage analysis of PMEL17 and Dominant white. By sequence analysis they showed that the Dominant white allele was associated with 9-bp insertion in exon 10, leading to an insertion of three amino acids in the PMEL17 transmembrane region.

1.6.7.1 Barring

Barring is the alternate transverse markings of two distinct colours on a feather. It may be regular or irregular depending on the breed (Dorshorst and Ashwell, 2009). As with most of the dominant mutations, the gene for barring is incompletely dominant. In other words, two such alleles can produce a greater effect than one. The allele for barring inhibits the deposition of melanin, thus causing white bars to be superimposed on a feather that would otherwise be black (Hutt, 1949). Dorshorst and Ashwell (2008) used gene mapping approach to isolate the sex-linked barring gene variant. They developed a mapping population consisting of 71 F_2 chickens from crossing a single Barred Plymouth Rock female with a White Crested Black Polish male. They used existing and novel microsatellite markers located on the chicken chromosome Z to genotype all individuals in mapping population. By single marker association analysis, it was revealed that a 2.8-Mb region of the distal q arm of chicken chromosome Z was significantly associated with the barring phenotype ($P < 0.001$). It was further analysed that the causal mutation is located within a 355-kb region showing complete association with the barring phenotype and containing 5 known genes [micro-RNA 31 (*miRNA-31*), methylthioadenosine phosphorylase (*MTAP*), cyclin-dependent kinase inhibitor 2B (*CDKN2B*), tripartite motif 36 (*TRIM36*), and protein geranylgeranyltransferase type I, β subunit (*PGGT1B*)], none of which have a defined role in normal melanocyte function.

1.6.7.2 Lacing, Tipped and Speckled feather

Laced feather have a border of contrasting colour around the entire web of a feather. Lacing is caused by autosomal recessive gene, *la*, in the homozygous state. Its effect

is influenced by a modifying gene. In tipped feathers, colour is eliminated from the tips of numerous of feather in all parts of the body. This pattern is also called spangling. Asmundson and Milne (1930 quoted by Hutt 1949) suggested this pattern is controlled by an autosomal recessive gene in the homozygous condition. In speckled feathers, patches of different colours occur on the whole area of the feather. This pattern is controlled by incompletely dominant autosomal gene. In birds, heterozygous for this trait, manifestation of speckling is more complete in males than in females (Hutt 1949).

1.7 Specific Objectives

This study was designed to find the genomic region controlling incubation behaviour in chicken both for the practical benefits this might bestow and the new biological information it would provide of a key behaviour. For this purpose, the main objective was

1. To test the hypothesis that genetic loci controlling incubation behaviour will be detectable by mapping and fine mapping in an F2 populations of chickens founded from White Leghorn and Silkie chickens (chapter 3 and 4).

The use of the Silkie has however provided a major opportunity to study the genetics of Mendelian traits which are not only important from a developmental point of view but also some have economic importance. This has already proven useful to characterise the trait of polydactyly (Dunn et al., 2011) . My second objective therefore was

2. To test the hypothesis that genetic loci controlling Morphological traits will be detectable by mapping and fine mapping in an F_2 population of chickens founded from White Leghorn and Silkie chickens (chapters 5 and chapter 6).

Finally I looked at comb type and fertility and my final objective was the exploration of candidate genes for comb type;

3. To test the hypothesis that Wnt genes are candidates for the genetic variation in comb type (chapter 7).

Chapter 2 Material and Methods

2.1 Introduction

This chapter details the resource population, data collection (incubation behaviour, morphological traits), marker information, methods of data analysis and generic laboratory techniques used in chapters 4-7 of the thesis. In addition further explanations are given in relevant chapters where details are specific to those chapters.

2.2 Resource population

Two divergent lines of chicken, White Leghorn (WL) and Silkie (SLK) were used to set up the F_2 cross used in the study. The White Leghorns were from a flock maintained at Roslin Institute and had 0% of incubation behaviour, single comb, normal feathering, no crest, clean legs and white skin. The Silkie were obtained from the Wernlas Collection (Shropshire, SYL 9BL) a certified rare breeds farm and were maintained at the institute. Incidence of incubation behaviour was 98% when tested and they possess rose comb, Silkie feathering, a crest, feathered legs, black skin and black internal organs. All mating was done by artificial insemination, Three WL sires were crossed with 8 SLK dams and 2 SLK sires were crossed with 10 WL dams in the F_0 generation (Figure 2-1). 4 males and 20 female from F1 (Figure 2-2) cross were used to establish the F2 population (Figure 2-3). Phenotypic data for incubation behaviour was successfully recorded from 280 F2 animals in 19 families.

Figure 2-1 In F_0 Population 3WLH ♂ were crossed with 8SLK ♀ (left side) and 2SLK ♂ x were crossed with 10 WLH♀ (right side)

Figure 2-2 In F1 population, male and female from WL ♂ x SLK ♀ were crossed (upper left and right) and male and female from SLK ♂ X WL♀ were crossed (lower left and right). A difference of comb colour in females is observed in the reciprocal cross.

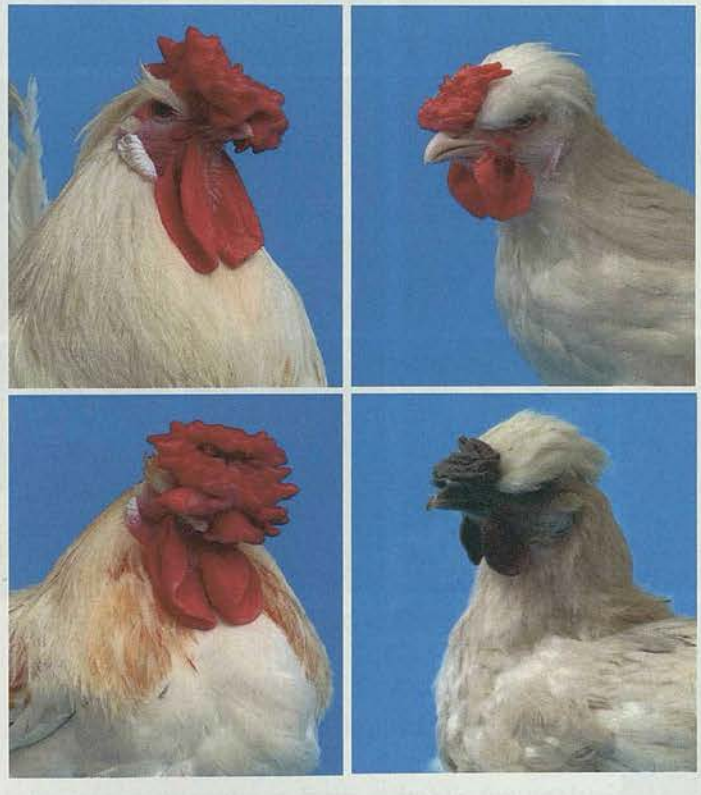
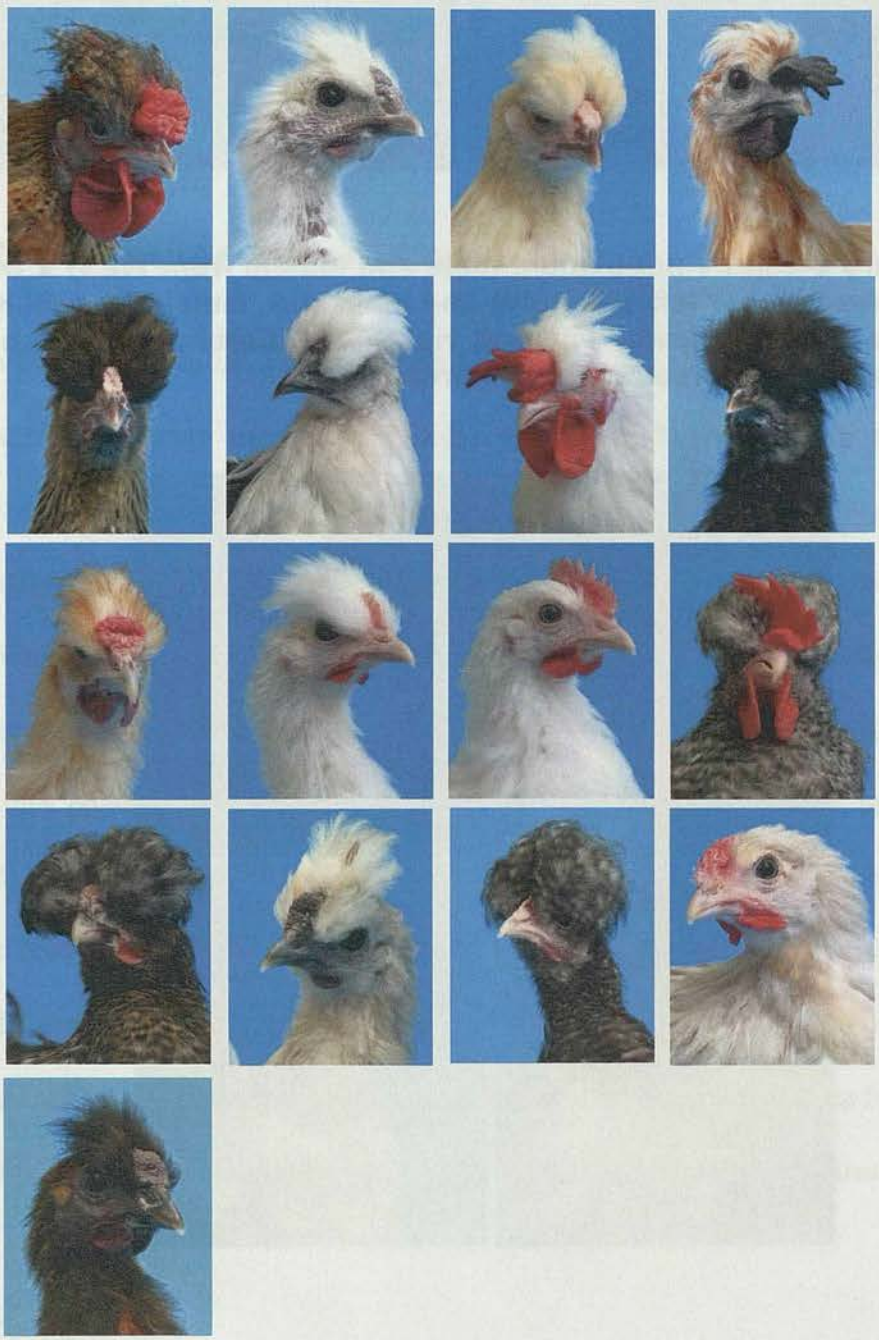


Figure 2-3 Birds of F_2 population of a cross of White Leghorn (WLH) and Silkie (SLK) showing different type of comb, crest, feather colours and feather patterns



2.3 Incubation phenotype

After hatching, the F1 and F2 generations were reared in floor pens on short days (8 hours light: 16 hours dark) for sixteen weeks. After sixteen weeks, the birds were transferred to new floor pens (4m x 1m) in groups of six or seven and reared on long days (16 hours light : 8 hours dark). Temperature was maintained between 18 to 23°C and the birds had access to food and water *ad libitum*. Each pen contained nest boxes, with wood shavings and hard-boiled eggs to encourage incubation behaviour. During daily behavioural observations, birds which demonstrated persistent nesting, raising their feathers when approached and clucking for a number of consecutive days were recorded as incubating, and any fresh laid eggs were removed from the pens. The birds were maintained in these conditions up to the age of one year. The number of days between entering the pen and the onset of incubation behaviour were recorded, varying between 61 to 140 days for the birds that exhibited incubation behaviour.

2.4 Morphological trait phenotype

All the Morphological traits, comb type, crest, feather type, feathered leg, skin colour and feather pattern were recorded at the end of experiment. For fibromelanosis of internal organs, 70 birds were killed at the end of experiment to measure the traits.

These traits were appraised in the light of the review of the literature and their known genetic inheritance to make a form suitable for analysis. Further detail of the traits and how the data was coded can be found in the respective chapters

2.5 Genotyping

Blood samples were collected from all the individuals of F_0, F_1 and F_2 and DNA was extracted as previously described (Dunn et al., 2009).

2.6 DNA Extraction

Following protocol was used for the DNA extraction in the current study

First Pipette 500 µl of DNAzol into a sterile (autoclaved) Eppendorf tube. Place 5-8 µl of whole blood in the lid of the Eppendorf, close and shake immediately. If clotted mix gently for 30 minutes to break up lumps. If large amounts of un-dissolved debris exist then centrifugation was performed at 10,000g for 10 minutes. After centrifugation, 250 µl of isopropanol was added to the tube and mixed gently and incubated at room temperature for 5 minutes. At this stage, DNA was visible, and centrifugation at 10,000g for 4 minutes was performed and the supernatant removed. The DNA was washed with 1ml of 70% Ethanol and centrifuged at 10,000g for 3 minutes then repeated. After removing the supernatant, the pellet was air dried (not too dry, only for 10-15 sec) and resuspend in 50 µl of 8mM Sodium Hydroxide overnight for mixing at 37oC. After overnight mixing with sodium hydroxide, 300-450 µl of TE Buffer was added in it and heat it at 50°G and mix gently. Optical Density of DNA was measured to determine the concentration of DNA obtained. DNA samples were stored at 4oC.

2.6.1 Microsatellite markers

A total of 90 microsatellite markers covering 23 autosomal linkage groups and sex chromosomes were genotyped in the F_0, F_1 and F_2 population (Table 3-3). These markers were known to be informative from an initial screen and their spread was across the known linkage groups. Fragment sizes were determined using GENESCAN 3.1 DNA fragment analysis and GENOTYPER 2.1 (PE Biosystems, Foster City, USA).

2.6.2 SNPs markers

After initial analysis for potential QTLs, the number of markers was increased on chromosomes (2, 5, 7, 9, 13, E22C19W28 and Z) and linkage groups which were not represented were added (19, 20, 21, 22, 24, 25, LGE64) by genotyping the entire population for 384 SNPs (Table 4-4). The SNP markers were selected from the Ensembl genome browser (http://www.ensembl.org/Gallus_gallus/Info/Index) and a list of validated SNPs (Groenen et al., 2009). The SNP markers were genotyped by Golden gate Vera code genotyping assay (Illumina) and Bead Studio software was used to analyse SNP data and as a first pass to remove bad or un-informative markers. Out of 384 SNPs markers, 218 SNPs markers were informative and of suitable quality. After analysing these SNP markers, an additional 32 SNPs were added in the peak region of the best QTL on chromosome 5. These SNPs markers were obtained by comparing the genome sequence of Silkie (SLK) and White Leghorn (WLH) using solexa (Illumina) next generation sequencing.

2.7 Software used

All Pedigree information, marker genotypes and traits phenotypic data was stored in resSpecies (Law and Archibald, 2000). Each marker was checked for marker, individual and pedigree errors using the related Genotype checker software (Paterson and Law, 2011) prior to submission.

2.8 Map construction

Crimap software was used for the construction of a linkage map for each linkage group (http://saf.bio.caltech.edu/saf_manual/crimap-doc.html). It calculates the distance between markers on the basis of recombination fraction and the intensity of linkage of each marker with one another on the same linkage group as well as with other linkage group on the basis of LOD score.

2.9 QTL mapping

Genotypic and phenotypic data for each trait was exported from the resSpecies database. The interval mapping method (Haley et al., 1994) for QTL analysis was carried out using GridQTL which is a grid-based portal version of the QTL Express program ((http://gridqt1.cap.ed.ac.uk:8080/gridsphere/gridsphere?cid)(Seaton et al., 2002).

The additive effect is modelled as half of the difference between Line 2 and Line 1 genotypes at the QTL, i.e.

$$a = (QQ \text{ (line 1)} - qq \text{ (line 2)})/ 2,$$

A positive value indicates that the increasing allele originates from Line 1i.e from white leghorn.

The dominance effect is defined as the deviation of the heterozygous animals from the mean of the two types of homozygous animals.

$$d = Qq - \tfrac{1}{2}(QQ + qq),$$

A positive value indicates that the heterozygote have greater effect than the midparent.

The analysis was carried out by using the BCF2 portlets. Family, Pen and pen year was used as fixed effect under dominance and additive effect model for the trait of incubation status and for each of the cumulative periods. F-statistic profiles were generated at 1 cM intervals

2.10 Determination of significance thresholds

In single and two QTL detection, significance thresholds were determined by conducting 5000 permutations (Churchill and Doerge, 1994) and 1000 bootstraps were used to generate 95% confidence intervals (Lander and Botstein, 1989, Visscher et al., 1996). A QTL was considered as being significant if it had an F value greater than the $P \leq 0.05$ experiment-wide threshold value and highly significant if the F value exceeded the $P \leq 0.01$ threshold (Kruglyak and Lander, 1995). Alternatively the QTL was considered to be suggestive if it had an F value exceeding the $P \leq 0.05$ chromosome-wide threshold.

2.11 Extraction of Ribonucleic Acid (RNA) using ultraspec II method

Embryos of stage 19 and 20 were rapidly dissected with sterile dissection instruments, weighed and placed in RNAase-free eppendorfs which were snap frozen in liquid nitrogen and stored at -80°C.

The embryos were placed in matrix D tubes containing 1000µl of Ultraspec II (Biotecx). Homogenisation was done to disrupt the tissue architecture of embryos and release the RNA with a FastPrep FP120 homogeniser using two 20-second pulses at speed 6. After 5 minute incubation on ice to allow the complete dissociation of nucleoprotein complexes, 200µl of chloroform was added to separate the aqueous and organic phases. The solution was then inverted by hand for 15 seconds incubated on ice for 15 minutes and then inverted again to ensure isolation of RNA molecules. The solution was centrifuged at 13000K for 15 minutes at 4^0C to separate the RNA from tissue debris, DNA and proteins. 500 µl of the clear upper aqueous layer supernatant which contains the RNA was transferred into 1.5 ml Eppendorf. 250µl of isopropanol and 25µl of RNA TackTM Resin (Biotecx) were added in it and vortexed for 30 seconds. The precipitated total RNA attached to the RNA Tack Resin was then separated from the solution by centrifugation of the tube at 13000K for 5 minutes. The supernatant was removed immediately and the RNA pellet was washed 2 times 1 ml of 70% EtOH. After each wash the solution was centrifuged at 13000 K for 5 minutes. All residual EtOH was removed from the tube, and the total RNA attached to the RNA TackTM Resin was air dried in a

desiccator for approximately 45 minutes. The RNA TackTM Resin was re-suspended in 30µl of RNAse free H20 and stored at –80°C.

2.11.1 RNA quantification

The quality and the quantity of the RNA extracted was analysed by measuring the optical density (OD) at 260nm and 280nm of a 1:50 dilution of the sample. The ratio of the OD260/OD280 is an indication of the purity of the RNA and can be compared with pure RNA (ratio =2.1).

Ratio = (OD260 / OD280)

Quantity = (OD260 x 40 x 50) / 1000 = total RNA $\mu g.\mu l^{-1}$

2.12 Reverse transcription of RNA to single stranded cDNA

Reverse transcription of total RNA was done using a First Strand synthesis kit (GE Healthcare) to synthesise single stranded complementary deoxyribonucleic acid (cDNA). 1µg of total RNA made up to 4µl by adding the appropriate amount of RNAse free H_20. The total RNA was then heated to 65°C for 10 minutes. The RNA was then chilled on ice, as rapid cooling prevents the formation of secondary structure. The first strand cDNA synthesis kit components listed below were used for making a master mix.

1st strand synthesis mix = 2.5µl

200mM Dithiothreitol (DTT) = 0.5µl

0.2 µg/µl Not I-d$(T)_{18}$ bifunctional primer = 0.5µl

Add this 3.4 µl master mix in 4 µl of RNA. Master mix can be prepared accordingly depending upon the number of samples. The reaction mix was then incubated at 37°C for 1hour. A final incubation at 90°C for 5 minutes was done to denature the reverse transcriptase enzyme. Samples of cDNA were stored at -20°C for long term storage.

2.13 cDNA amplification using PCR

For the amplification of cDNA of WNT6 and WNT10A, master mix was prepared for a 40 µl reaction. For one reaction, 1 µl of forward primer and 1ul reverse primer were added along with 4.0 µl of 10XdNTPs, 4 µl of 10X Mg+2 , 0.2 µl of Fast start Taq, 25.8 µl of MQ water. Out of this 36 µl l master mix 4ul cDNA was added to make the volume up to 40 µl. The amount of master mix was prepared depending upon the number of samples. cDNA was amplified using standard PCR conditions (initial denaturation step of 95°C/5minutes, 35 cycles of denaturation 95°C/30s, annealing 61.9°C/30s and elongation 72°C/90s and a final extension 72°C/7minutes). The PCR products were visualised using electrophoresis through a 2% agarose gel.

2.14 Cloning the WNT6 and WNT10A cDNA into pBluescript II SK(+) vector

2.14.1 Excision and Purification of DNA from gel

After amplification of the desired PCR product, blunt ending of the PCR product was done with the addition of 10% by volume of DNA polymerase I (Klenow fragment) (Promega) and incubated it at 37°C for 20 minutes. The blunt-ended PCR

product was then electrophoresed on a 2% agarose gel and excised. The DNA was extracted from the gel using the GeneClean® Spin Kit (Q-Biogene), according to the manufacturer's recommendations.

2.14.2 Linearisation of PBSK+ vector

0.2 µl of a pBluescript II SK (+) vector (PBSK+) was linearised with 1.0 µl *EcoR*V along with 1.0 µl Buffer B and 8.7 µl of H_2O. A final dilution of the reaction mix by the addition of 30µl H_2O was performed to give a final plasmid concentration of 5ng.µl^{-1}.

2.14.3 Ligation

A rapid DNA ligation kit (Roche Applied Science) was used to insert the DNA into the vector. The components listed below were added to give a 20µl total volume reaction mix.

~0.4 µg.µl-1 purified PCR product = 7 µl

Linearised 5ng.µl-1 pBSK+ vector = 1 µl

5x DNA dilution buffer = 1 µl

T4 DNA ligase = 1 µl

2x T4 DNA ligation buffer = 10 µl

The reaction mix was allowed to incubate at room temperature for 5 minutes. An additional *EcoR*V digest was done after ligation to eliminate any plasmid without insert. The tubes were then chilled on ice prior to transformation.

2.14.4 Transformation

The ligation reaction was transformed into XL1 Blue E. coli competent cells (Agilent). The cells are prepared according to manufacturer instructions. The ligation

reaction was added to a 50µl aliquot of the competent cells and incubated on ice for 30 minutes. The cells were heat shocked for 45 seconds at 42°C and immediately incubated on ice for 2 minutes. 0.9 ml of pre heated SOC was added to each sample and incubated them at 37°C for one hour with shaking at 225-250 rpm. The cells were then plated onto pre-warmed agar plates and incubated at 37°C for overnight. Colonies were picked after that and grown in LB broth. The Plasmid DNA purification was done using the QIAprep Miniprep Kit according to the manufacturer's instructions. The selected clone was grown for plasmid DNA purification using the HiSpeed Plasmid Maxi Kit according to the manufacturer's instructions.

2.15 *In-situ* Hybridization Digoxygenin (DIG)-labelled Probe synthesis using T7 and T3 RNA polymerase

The cDNA of WNT10A and WNT6 in pBSK (+) vector was linearised with *BamH*I and *Not*1 restriction endonucleases. 1µg of the plasmid was used to synthesis RNA run-off transcripts by using T3 (WNT6) and T7 (WNT10A) RNAse polymerase, by adding the 4 µl of 5 x Transcription buffer, 2 µl DTT, 2 µl of RNA labelling mix – DIG, 0.5 µl of RNAse inhibitor and adjusting the total reaction volume to 20µl with RNAse free H20. Then this reaction is incubated for four hours at 37°C. The probes were then purified using Probe Quant G-50 Micro Columns (GE Healthcare) according to the manufacturer's instructions. The quality and the quantity of the RNA synthesised was analysed by measuring the optical density (OD) at 260nm and 280nm and by electrophoresis on a 2% agarose gel.

2.15.1 Dissection, fixation and dehydration of embryos for *in- situ* Hybridization

Embryos of different stages (20 HH, 21 HH, 23 HH and 25 HH) were dissected with RNAse free instruments and immediately placed in 1x phosphate buffered saline (PBS) (pH 7.4). After clearing the embryo from the surrounding membrane, they were placed in a 4% paraformaldehyde (PFA)/PBS (pH 7.4) and maintained at 4°C overnight. For dehydration, the embryos were washed with a series of 10 minute washes twice in 1 x PBT buffer (PBS and 0.1% Tween 20), 25% MeOH/PBT, 50% MeOH/PBT, 75% MeOH/PBT and finally twice in 100% MeOH. The embryos were then stored at -20°C.

2.15.2 Permeabilisation

The embryos were washed 2 x 5 minutes with 4°C 50% MeOH PBT (50% MeOH , 50% PBT) and then again 2 x 5 minutes washing with 100% PBT . These embryos were then treated with proteinase K diluted 1: 1000 with PBT for 1 minute per stage. (This is the critical step). The samples were given 3 x 5 minute washes in PBT. Two times rinsing were done quickly with PBT. The embryos were refixed with 4 % paraformaldehyde and 0.2 % glutaraldehyde for 20 minutes on ice. The samples were then subjected to 3 x 10 minute washes in PBT.

2.15.3 Pre-Hybridization and Hybridization

The embryos were placed in pre-Hybridization solution (50% formamide, 5 x SSC, 2% Blocking Reagent (less than 2% maelic acid) (Roche Applied Science), 0.1% Trition-X-100, 0.1% CHAPS (VWR), 20µg/ml tRNA (Boehringer), 5mM EDTA,

50µg/ml Heparin (Sigma-Aldrich) overnight at 65°C, and then hybridised for 2-3 days in Hybridization solution (50% formamide, 5 x SSC, 2% Blocking Reagent (Roche), 0.1% Trition X, 0.1% CHAPS, 20µg/ml tRNA, 5mM EDTA, 50µg/ml Heparin with a minimum of 20ul probes in 1.5 ml of Hybridization buffer.

2.15.4 Immuno-detection with Anti-DIG antibody

The embryos were washed following Hybridization. The following series of washes was done, two washes in 2 x SSC each for 10 minutes at 70°C, three washes in 2 x SSC/0.1% CHAPS each for 20 minutes at 70°C, three washes in 0.2 x SSC/0.1% CHAPS each for 20 minutes at 70°C and two final 10 minute washes in KTBT (50mM Tris-HCL pH7.5, 150mM NaCl, 10mM KCl, 1% TritonX-100) at room temperature. The embryos were blocked in 20% heat inactivated FCS/KTBT for 3 hours at 4°C and then incubated in a 1:1000 dilution of mouse anti-DIG-AP Fab fragment (Boehringer) in 20% heat inactivated FCS/KTBT overnight at 4°C. The embryos were then washed 5-6 times with KTBT (I hour) at room temperature and left at 4°C overnight in KTBT.

2.15.5 Colour detection

The embryos were washed two time each for 15 minutes in freshly made NTMT (100mM Tris-HCl pH9.5, 50mM MgCl, 100mM NaCl, 0.1% TritonX-100) at room temperature. In the dark, add 3.5µl/ml nitro-blue tetrazolium chloride (NBT) (Sigma-Aldrich) and 3.5µl/ml 5-bromo-4cholor-3-indolyl-posphate (BCIP) (Sigma-Aldrich) in NTMT. Once the desired staining was achieved, the embryos were fixed in 4% formaldehyde/PBS.

2.16 Western blotting of WNT6

2.16.1 Dissection of embryos and sample preparation

The embryos of White Leghorn and Silkie at stage 20-21 were rapidly dissected with sterile dissection instruments and were placed in cold PBS buffer. Head of these embryos were cut separately for both of these breeds. These tissues were weighed and chopped into pieces and place on dry ice. One Proteinase inhibitor tablet was added in 10 ml of 1X Rippa (Radio Immuno Precipitation Assay buffer) buffer (millimore). 100ul of this buffer was added in 2.5mg of tissues. These samples were ground in sonicator 2 x 20 seconds with wait of 1 minute on ice. These samples were rotated at 4^0C for 20 minutes and then centrifuged at 11000rpm for 20 minutes at 4°C. Supernatants was removed and added to a fresh Eppendorf and stored at -20 ^{0}C. The concentration of Protein was determined by using BCA protein Assay kit (Thermo scientific). By using this kit, the concentration of Protein is measured in mg/ml. The protein concentration of sample were Silkie (0.40 mg/ml) and White Leghorn (0.70 mg/ml)

2.16.2 Protein Precipitation and Separation

The samples were mixed with NuPAGE® LDS Sample Buffer (4X) and Nupage reducing agent (10X). For each well of gel, sample was prepared with the following concentration. White Leghorn (4.7µl sample, 5 µl sample buffer, 2.5 µl reducing agent and 15.3 µl H_2O), Silkie (20 µl sample, 5 µl sample buffer, 2.5 µl reducing agent), White Leghorn /Silkie (10.5 µl sample, 5 µl sample buffer, 2.5 µl reducing agent and 9.5 µl H_2O). These samples were kept at 70 ^{0}C for 10 minutes. 1 X running

buffer was prepared (Add 50ml 20X NuPAGE® MES running buffer to 90 ml of water to prepare 1X SDS running buffer). NuPAGE® Bis-Tris Gels 10% or 4-12% gel was used. The gel was fixed in Invitrogen electrophoresis tank. The upper (200ml) and lower chamber (600 ml) of the electrophoresis system were filled with 1X running buffer. 500ul of NuPAGE® antioxidant was mixed in remaining 200 ml 1X running buffer and added in the upper chamber. The protein samples of appropriate concentration were loaded into gel. The gel was run at 200V for 35 minutes.

2.16.3 Transfer of Protein and staining

After running gel, protein were wet transferred onto PVDF (Odyssey® Western Blotting Kits LT, Licor) membrane in X_cell surlock blotting module. The PVDF membrane was pre wet for 30 seconds in methanol and briefly rinsed in deionized water and then membrane was placed in a shallow dish containing 50-100 ml transfer buffer for several minutes.

After removing gel from the cassette, the surface of the gel was made wet with the transfer buffer and pre-soaked transfer membrane was placed on the gel. Air bubbles were removed by rolling a glass pipette over the membrane surface. The pre-soaked filter paper in 1X transfer buffer was placed on top of the transfer membrane and one on the bottom of the gel. Remove any trapped air bubbles. On both side, around the filter papers, two pre-soaked pad were placed and arranged them in the blotting module. IX transfer buffer was poured in the inner side. Transformation of protein onto membrane was done at 30V for 1 hour. After this, membrane was transferred into 5 ml Odyssey buffer (Licor) for one hour for blotting. 60 µl Primary antibodies

of Rabbit anti-WNT6 (Invitrogen) and 5 µl of mouse anti-gamma tubulin (Invitrogen) was added in 5 ml of Odyssey buffer (Licor) and membrane was incubated overnight at 4 ^{0}C. 3 times, 15 minutes washing was given with PBST to remove the antibodies. In 5 ml of odyssey buffer, 3 µl of anti-mouse IRDye, 800 CW and 3 µl of IRDye 800CW Goat Anti-Mouse Secondary Antibody (25 µl, 1 µg/µl) and 3 µl of IRDye 680RD Goat Anti-Rabbit were added and membrane was placed in it for one hour. Membrane is washed again with PBT, 3 times for 5 minutes and finally rinsed in PBS. For protein detection, membrane is analysed in a Li-COR (Odyssey) infra-red scanner (version 3).

Chapter 3 Mapping of Incubation Behaviour (Broodiness) Trait

3.1 Introduction

Substantial advances have been made over the past decades through the application of molecular genetics in the identification of chromosomal regions that contain loci that affect traits of importance in livestock production (Andersson, 2001). This discovery provides opportunities to enhance genetic improvement programs in livestock by direct selection on genes or genomic regions that affect economic traits through marker-assisted selection and gene introgression.

The sequencing of chicken genome and its subsequent release in the public domain in 2004 propelled the poultry breeding industry into the genomic era. This genomic information is actively used to enhance the breeding programs and improve the selection efficiencies (Fulton, 2012). Identifying genomic regions through QTL and genome wide association analysis is important to understand the domestication of chicken as well as to understand molecular pathways underlying phenotypic traits and breeding goal (Elferink et al., 2012). These developments in genomics have provided opportunities to improve breeding programs in poultry by genotyping large number of genetic markers across the genome(Ordas, 2012).

3.1.1 QTL mapping in chicken

QTL mapping studies in the chicken have been used extensively to identify the chromosomal regions that contribute to variation in economically important traits. The ultimate goal of these studies in chicken is to identify genetic markers that are close to the QTL [linkage disequilibrium (LD) markers] or the gene underlying the QTL (direct marker)(Abasht et al., 2006, Dekkers, 2004). The emergence of

molecular genetics spurred efforts to identify the chromosomal regions, genes and mutations underlying the phenotypic variations (Georges, 2007). Different experimental lines of chickens have been created to increase our understanding of genetic control over important production traits like body weight, growth, body composition, egg production and disease resistance (Hocking, 2005). Early QTL mapping studies were performed with sample sizes in the order of hundreds of individuals and approximately 100 molecular markers and, for most traits, consistently detected few QTLs with moderately large effects (Hocking, 2005, Abasht et al., 2006)

In the context of the study presented in this thesis, QTL mapping has been used to find out more about the actual genetic nature of trait and also to identify the chromosomal regions that control the trait of incubation behaviour. It is also hoped that the results will help to understand the process of domestication since the loss of incubation behaviour would seem to be potentially an important step in the domestication of poultry concomitant on the development of artificial incubation techniques.

Incubation behaviour, often known as broodiness, is a facet of maternal behaviour and is a complex trait. During domestication incubation behaviour has been eliminated completely in certain breeds of chicken, notably the White Leghorn but still exists in many breeds of poultry and of course is present in the wild populations that the domestic chicken is derived from such as the Red Jungle fowl (Collias and Collias, 1967).

For the current QTL mapping study of incubation behaviour, two breeds were used. The White Leghorn (WL), a breed of the Mediterranean class which has high egg production and low or no incidence of incubation behaviour (Hutt, 1949). There has been a belief that a major gene may be responsible for this disappearance of incubation behaviour (Romanov et al., 2002, Zhou et al., 2008, Sharp, 2009). On the other hand, the Silkie breed, apparently bred mainly for ornamental purposes, carries genes for many morphological traits including polydactyly (Po), Silkie feathering (h), fibromelanosis (Fm) and rose comb (R) (Hutt, 1949) and in the context of this chapter it has a high incidence of incubation behaviour (Liang et al., 2006). The Silkie breed belongs to the Asiatic class and is thought to have originated in China (van Wulfften Palthe, 1992).

Solving the molecular mechanism of incubation behaviour will allow the improvement of egg production by selection or introgression of the loci into lines of hens favoured by farmers and adapted to local conditions. Incubation often persists in these lines and is an impediment to optimal production

3.2 Objective

To test the hypothesis that genetic loci controlling incubation behaviour will be detectable by mapping in an F2 populations of chickens founded from WL and Silkie chickens.

3.3 Material and Methods

3.3.1 Resource population

Detail of resource population is described in chapter 2, section 2.2.

3.3.2 Phenotypic data collection

The details for phenotypic data collection on incubation behaviour is shown in chapter 2, section 2.3.

3.3.3 Data treatment for analysis

3.3.3.1 Incubation behaviour

Data for analysis was prepared in 2 ways. Firstly the data was categorised on a 3 point scale so that all birds which had shown clear symptoms of incubation behaviour were categorised as incubating category 1 and those which showed some signs of incubation behaviour but not persistent sitting as category 2 and finally the 3rd category were those birds which showed no signs of incubation behaviour. The first category included birds who showed full signs of incubation behaviour including vocalisation, aggressive behaviour, sitting on the eggs; the 2nd category included birds that showed some signs, for example vocalisation and/or aggression. Only incubation behaviour was observed for those birds that lay number of eggs or having number of clutch.

3.3.3.2 Early Incubation behaviour

In the second approach the data was split into cumulative time periods because we believed that the time it takes hens to commence incubation behaviour might reveal information on the strength of motivation to incubate. The same approach as above was taken except the data was considered as a bivariate trait with category 1 birds as the 1st category and birds in category 2 and 3 forming the 2nd category. The experiment was split into 5 cumulative time periods, by categorising and analysing birds that showed full signs of incubation behaviour from 25 to 30, 25 to 36, 25 to 42, 25 to 48 and 25 to 53 weeks of age. A period of five weeks gave a sufficient period to get enough observations of incubation behaviour to avoid problems of bias due to too few individuals in that observation class.

3.3.4 Power and Precision of F2 Population

For the trait of incubation behaviour, 280 individual of F2 populations were studied. An F2 population of 280 individuals would have a 95% chance of detecting a QTL that explains 5.5 % of phenotypic variance.
(http://www.extension.org/pages/32355/equation used to-estimate-sample-size-required-for-qtl-detection).

3.3.5 Genotyping

Blood samples were collected from all the individuals of F_0, F_1 and F_2. DNA was extracted by using the method described in chapter 2, section 2.5.1.

3.3.6 Map construction using Crimap

Firstly a map for the microsatellite markers was created based on the position of markers in the chicken genome assembly (build 2.0) although there were genetic positions already recorded in resSpecies. This was to re allocate any markers that might have been misplaced. For construction of the map, the following steps were used.

1. A marker's starting and ending position on each linkage group was noted from (http://www.ensembl.org/Gallus_gallus/Info/Index).
2. Crimap software was used to map markers on each linkage group. Marker positions were fixed on each linkage group by using the "fixed" option. This option fixed the positions of markers by using the recombination fraction between any pair of adjacent loci. On each linkage group, difference in position between markers (cM) was found.
3. To check the linkage of each marker with one another on the same linkage group and on different linkage group, two-point analysis was carried out in CRIMAP. The two-point option of CRIMAP was used for two-point linkage analysis in which the recombination rate between each of the two most informative linked markers of a chromosome and each one of the other markers was estimated
4. To check the locus order of markers on each linkage group, "flipsn" was run in CRIMAP. If any negative value was found then marker on that linkage group was reordered and again "flipsn" was run to confirm the exact ordering. By this "flipsn" analysis for each linkage group, only linkage group

4 showed negative value of relative log10_likelihood value. So markers on this linkage group were reordered accordingly and then fixed at the optimal position.

5. The final map was created for use in analysis (Table 3-3).

3.3.7 QTL Analysis

For the purpose of analysis, genotype and phenotype data for each trait were exported from the resSpecies database after selecting the markers in Table 3-3. The linkage map also in Table 3-3 was used in the analysis. In interval mapping the intervals between pairs of flanking markers are tested in turn for evidence of the presence of a QTL at specific positions or intervals between the markers. In this case the interval was 2 cM. The interval mapping method for QTL analysis (Haley et al., 1994) was conducted using GridQTL which is a grid-based portal (Seaton et al., 2002) version of the QTL Express program. Grid QTL enable us to search for QTL, using a wide range of models from F2 crosses to general populations, with fast computation.

In Grid- QTL, the module used for analysis was BCF2 (Back cross F2 or F2 generation algorithm). For the analysis under this module, marker data are supplied for grandparents, (F1) parents and F2 progeny, and phenotypes are supplied for F2 individuals. The method was developed for inbred lines by Haley and Knott (1992) and extended for outbred lines by Haley et al. (1994)

The QTL analysis proceeds in two steps. First data on marker positions together with actual marker genotypes are used to calculate probabilities of individuals inheriting

0, 1 or 2 alleles from each of the two founder lines at positions through the genome and the parent-of-origin probability of the alleles. These probabilities are combined into "coefficients" that can be used to look at marker information content or marker segregation distortion. These coefficients are linear combinations of the conditional probabilities of line-origin (and parent-of-origin) given the marker data. In the second step of the QTL analysis the phenotypic data is regressed onto these coefficients. The simple nature of the regression approach means it is possible to fit various genetic and environmental models, such as one or two linked QTL, additive, dominance and parent-of-origin (e.g., imprinting) effects of QTL, effects of environmental factors (fixed effects) or covariates, interactions of QTL with fixed effects, etc.

The results of the chosen QTL analysis include the estimate of the model parameters (additive, dominance), the fitted fixed effects and the sums of squares of the full and reduced models.

The additive effect is modelled as half of the difference between Line 2 and Line 1 genotypes at the QTL, i.e.

$$a = (QQ(\text{line } 1) - qq(\text{line } 2))/ 2,$$

A positive value indicates that the increasing allele originates from Line 1.

The dominance effect is defined as

$$d= Qq - ½ (QQ + qq),$$

A positive value indicates that the heterozygote is larger than the midparent.

In Grid QTL, genotype, phenotype and map files were uploaded. The BCF2 Portlet in Grid QTL was used for the analysis. Initially a one-QTL search was performed under an additive and dominance model with no permutation for significant or suggestive evidence of QTL. In the case of incubation behaviour and early incubation behaviour, family and pen were used as fixed effect for an additive and dominance model. The 95% CI of the QTL position was determined with thousand iterations under bootstrap with resampling. Chromosomal wide and experimental wide permutation was performed with a thousand iterations. A QTL that achieved an F ratio exceeding the $P \leq 0.05$ chromosomal wide threshold was considered to be suggestive (Lander and Kruglyak, 1995). Significant QTL were fitted as background effects under additive and dominance model to observe the appearance of any other significant QTL.

3.3.8 Proportion of phenotypic variance explained by QTL

Under the additive-dominant model, the proportion of F2 phenotypic variance explained by the QTL is calculated by following formula:

% Phenotypic variance = (RRSS – FRSS)/RRSS) *100

RRSS is the residual sum of square from the residual model in which all the effects including background QTL effects are fitted but the QTL is omitted. The FRSS is the residual sum of square from the full model in which all the effects and the QTL fitted.

3.4 Results

3.4.1 Linkage Map

The CRIMAP software was used to map microsatellite markers on each linkage group. Initially, the map was created based on the position of markers in the chicken genome assembly (build 2.0). To check the linkage of each marker with one another on the same linkage group and on different linkage groups, two point analysis was performed.

1. During two point analysis, one exceptional case was found that had not been observed before. Marker MCW0056 which was mapped on linkage group 2 showed linkage with marker ADL0209 on linkage group 10.
2. During this analysis it was found that HUJ0008 belonged to linkage group 6, initially it was mapped to linkage group C21. The overall map length was increased to 22.1cM from 7.4 cM when this marker was added to the chromosome 6 map. This marker showed linkage with the first marker ADL0138 on chromosome 6 with map distance of 14.7cM.
3. It was found that markers on linkage group 3 and 4 showed different positions on the map, so accordingly markers were reordered on these linkage groups for analysis.

Regarding marker MCW0056, there is no evidence (http://www.thearkdb.org/) in the literature that this marker was linked with linkage group 10, so its position was not changed. Reordered markers on chromosome 3 and 4 were fixed by using the fixed

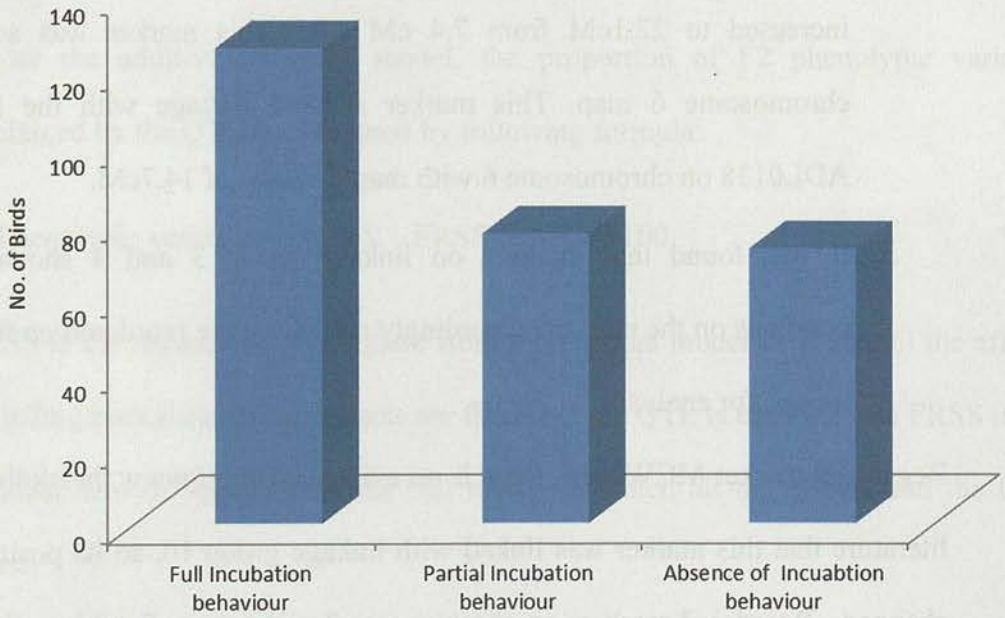
option in CRIMAP. Marker HUJ0008 which was previously mapped on linkage group C21 was fixed on chromosome 6.

3.4.2 Summary statistics

3.4.2.1 Incubation status

Test statistics for incubation behaviour showed that out of 276 birds studied for incubation behaviour in the F2 generation, 46% of birds showed full incubation behaviour, 28% of birds showed partial incubation behaviour and 26 % of birds showed no sign of incubation behaviour. Overall 72% of birds showed some signs of incubation behaviour (Figure 3-1).

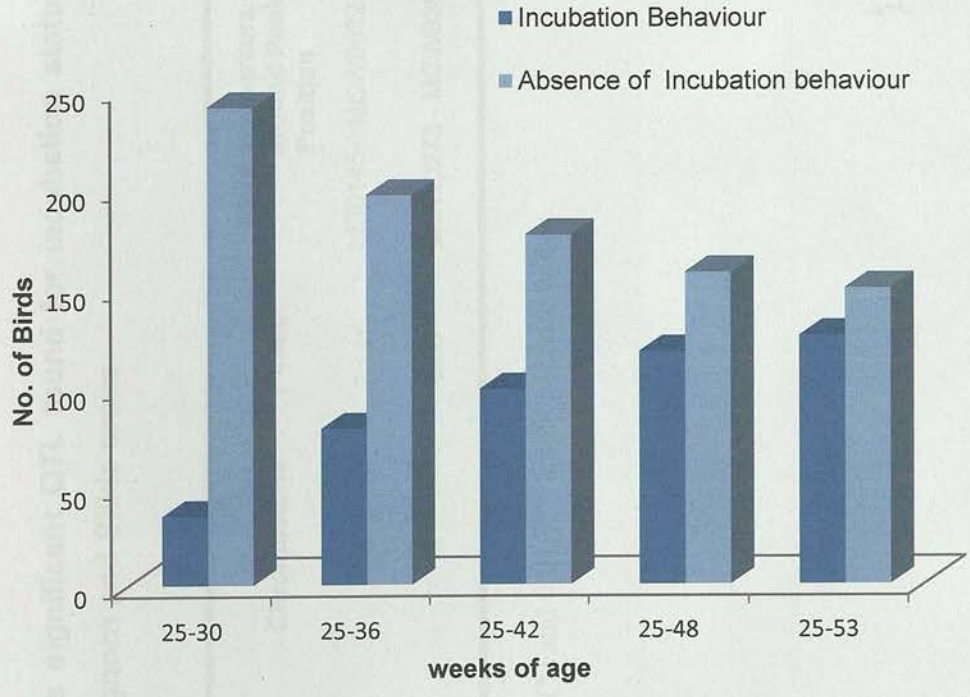
Figure 3-1 Summary statistics of birds showing full incubation behaviour, partial incubation behaviour and absence of incubation behaviour in whole recording period (one year)



3.4.2.2 Early Incubation behaviour

For the early incubation, data was split into cumulative time periods because we believed that the time it takes hens to commence incubation behaviour might reveal information on the strength of motivation to incubate. The experiment was split into 5 cumulative time periods, by categorising and analysing birds that showed full signs of incubation behaviour from 25 to 30, 25 to 36, 25 to 42, 25 to 48 and 25 to 53 weeks of age (Figure 3-2).

Figure 3-2 Summary statistics of birds showing incubation behaviour and absence of incubation behaviour in cumulative time periods from 25 to 30, 25 to 36, 25 to 42, 25 to 48 and 25 to 53 weeks of age



3.4.3 Significance of results

Hens tested in the same environment as the F_0 from the White Leghorn and Silkie had 0 and 90.5% incidence of incubation behaviour respectively. The F_1 generation hens showed the highest level of recorded incubation behaviour at 97%.

Genome wide significant QTL found for incubation status and early incubation behaviour in cross of White Leghorn (WLH) and Silkie (SLK) are presented in Table 3-1.

Additive and dominance effects of Incubation status and early incubation behaviour traits in the F2 cross of White Leghorn (WLH) and Silkie (SLK) are given in Table 3-2.

Table 3-1 Genome wide significant QTL found for Incubation status and early incubation behaviour traits in F2 population of White Leghorn and Silkie cross

Trait	Chromosome	F-ratio[1]	Flanking markers around Peak Position	Confidence Interval	Position (cM)	Significance Level
Incubation status	5	7.11*	LEI0145- MCW0032	16.5 - 111.5	79cM	0.05
Early incubation[1]	8	8.86**	MCW0275 - MCW0305	0-115	18	0.01

[1]Significant at 0.05 (*) and 0.01(**) levels genome wide

Table 3-2 Additive and dominance effects of Incubation status and early incubation behaviour in F2 population of White Leghorn and Silkie cross

Trait	Chromosome	Position (cM)	Additive +S.E	Dominance +S.E	Phenotypic Variance[1] (%)
Incubation status	5	79	0.08 ± 0.08	0.49 ±0.13	6.13
Early Incubation	8	13	0.26 ± 0.07	0.28 ± 0.11	7.54

1Proportional decrease in the residual sums of squares by fitting the model with the QTL compared to the reduced model.

3.4.3.1 Incubation Status

The evidence for QTL affecting incubation status (incubation behaviour over the whole recording period i.e. 52 weeks) on chromosome 5 was strong ($P<0.05$) (Table 3-1, Figure 3-3). Position of QTL is at 79cM.

The 95% Confidence Interval (C.I) for incubation status spanned a region around 95 cM (16.5-111.5). The additive and dominance effect of QTL were 0.08 and 0.49 respectively (Table 3-2). The peak position of QTL was at 79cM between flanking markers LEI0145- MCW0032.

3.4.3.2 Early Incubation

The experiment was split into 5 cumulative time periods, by categorising and analysing birds that showed full signs of incubation behaviour from 25 to 30, 25 to 36, 25 to 42, 25 to 48 and 25 to 53 weeks of age.

The QTL for early incubation behaviour (25-30 weeks) was found on chromosome 8 at 13cM is significant at the genome wide 1% level (Table 3-1, Figure 3-4). The additive and dominance effect of QTL are 0.26 and 0.28 respectively (Table 3-2)

This QTL for the trait of early incubation behaviour is dominant as the additive effect is approximately equivalent to the dominant effect (Figure 3-4).

Figure 3-3 Plot of F-ratio versus relative QTL positions on chromosome 5 for incubation status (incubation behaviour recorded over 52 weeks). The relative position on linkage map is presented on x-axis and the F-ratio on y-axis

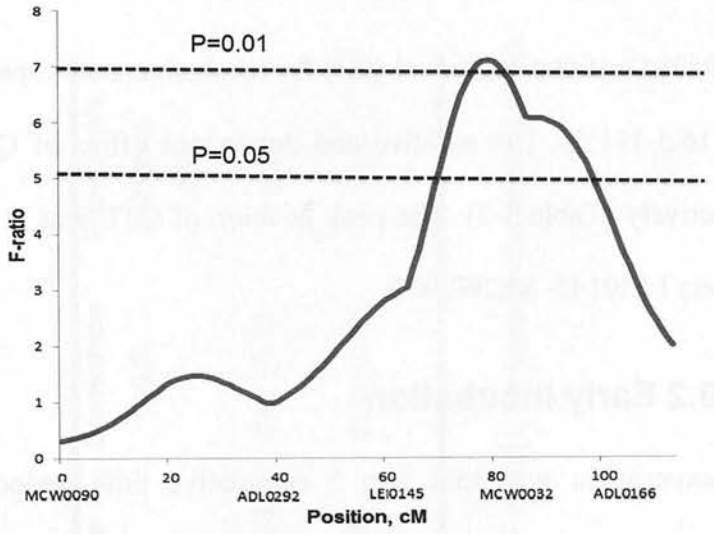


Figure 3-3a Graph showing additive and dominant effect of markers on chromosome 5

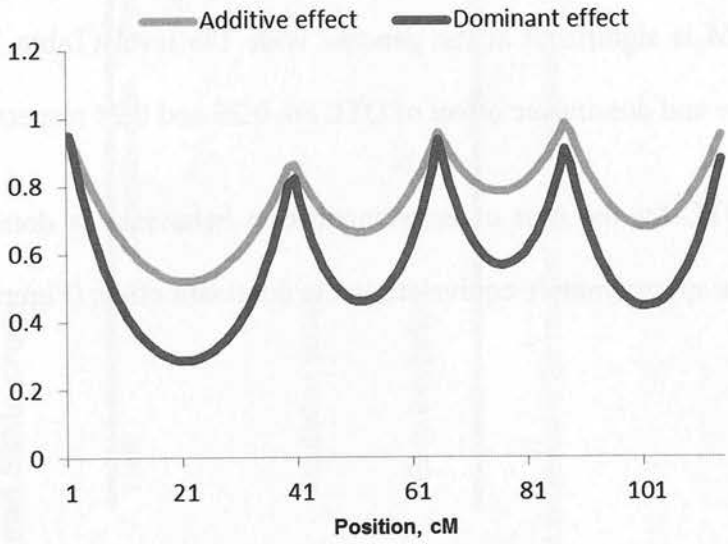


Figure 3-4 Plot of F-ratio versus relative QTL positions on chromosome 8 for early incubation (25-30 weeks). The relative position on linkage map is presented on x-axis and the F-ratio on y-axis

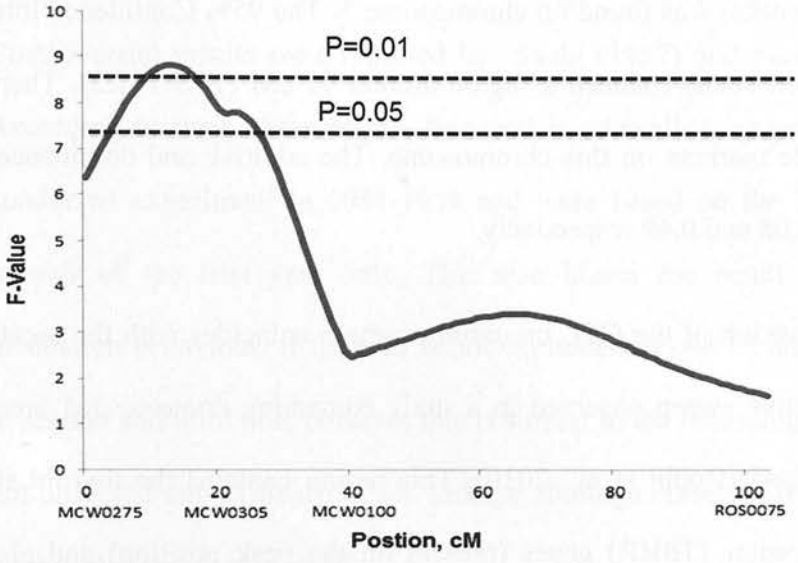
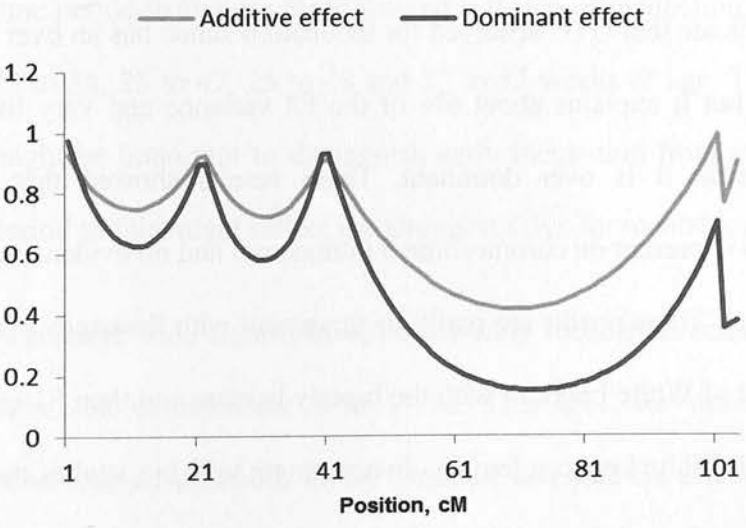


Figure 3-3a Graph showing additive and dominant effect of markers on chromosome 8



3.5 Discussion

One significant QTL ($P<0.05$) for incubation status covering the whole incubation period (52 weeks) was found on chromosome 5. The 95% Confidence Interval (C.I) for incubation status spanned a region around 95 cM (16.5-111.5). There are five microsatellite markers on this chromosome. The additive and dominance effect of QTL were 0.08 and 0.49 respectively.

The peak position of the QTL on chromosome 5 coincides with the location of the largest selective sweep observed in a study comparing domesticated breeds versus red jungle fowl (Rubin et al., 2010). This region contains the thyroid stimulating hormone receptor (TSHR) genes (present on the peak position) and also contain deiodinase, iodothyronine, type II and deiodinase, iodothyronine, type III (Dio2 and Dio3) which are enzymes responsible for thyroid hormone synthesis.

These results indicate that QTL observed for incubation status has an over dominant mode of action but it explains about 6% of the F2 variance and very little of the breed difference as it is over dominant. These results showed that QTL for incubation status is present on chromosome 5 (autosome) and no evidence was found on Z chromosome. These results are partly in agreement with Romanov et al. (1999) who used a cross of White Leghorn with the broody bantam and then F1 males were back crossed with White Leghorn female. In agreement with our studies they did not find any evidence of sex linkage of the trait but unlike this study concluded that two incompletely dominant autosomal genes are involved in the expression of the trait. In

the current study the locus on chromosome 5 is overdominant rather than incompletely dominant. Hay (1940) also did not find any evidence of sex linked genes for incubation behaviour in Rhode Island Reds and hypothesised the involvement of autosomal genes for the incubation behaviour trait.

Controversial results were reported by Saeki (1957) and Saeki and Inoue (1979). According to their observations, this trait is controlled by sex linked gene. Saeki conducted experiment in 1951-1954 and were based on the incubation behaviour records of the first year only. This also biases the result because of deferred incubation behaviour. If the trait is not expressed in one laying cycle but expressed in second and third one, however this is similar to the recording in this study. I have not observed any evidence of sex linkage although currently the number of markers on this chromosome is small.

Regarding early incubation behaviour, this is the first time data was split in different time period to analyse birds showed full sign of incubation behaviour from 25 to 30, 25 to 36, 25 to 42, 25 to 48 and 25 to 53 weeks of age. The hypothesis was that it might be important to distinguish early incubation from incubation over the whole period as this might reflect the strongest drive for incubation.

A genome wide significant QTL for early incubation behaviour (25-30 weeks) was found on chromosome 8 at 13cM. This QTL for the trait of early incubation behaviour showed both strong dominant and additive effect. (Table 3-2, Figure 3-4). Currently the number of microsatellite markers on chromosome 8 are 4. Peak position of QTL lies in between markers MCW0275 and MCW0305.

One QTL for incubation status on chromosome 5 and other QTL of early incubation on chromosome 8 supports the Romanov view to some extent that at least two genes are involved in the expression of the trait (Romanov et al., 2002) although in our case mode of inheritance is dominant rather than incomplete dominant (Table 3-2). Detailed discussion of QTL and genes is in the next chapters.

3.6 Conclusion

QTL for incubation status on chromosome 5 and QTL for early incubation behaviour on chromosome 8 appear to be potentially important. There is however a need to do further work to refine these QTL and to narrow down the confidence interval to try and reach the gene level. These QTLs are not only important from the point of view of reproduction as this trait controls egg production in the birds but the QTL on chromosome 5 may be important from the view point of domestication as this is the locus where a major selective sweep was observed(Rubin et al., 2010) .

To refine these QTL, a further strategy of fine mapping was adopted and is explained in the next chapter.

Table 3-3 Map of Microsatellite markers with genetic distance (cM) between markers on each linkage group used in the QTL analysis of the WLH and SLK F_2 population

Linkage group	No of Marker	Name of marker along with map distance(cM) between them on linkage group									
1	10	ROS0008	86.8	MCW0106	28.8	ADL0307	21.1	MCW0112	3.7	LEI0146	18.3
		LEI0101	7.8	LEI0071	22.4	LEI0079	3.5	ADL0314	67.2	ROS0025	
2	13	LEI0163	1.0	MCW0205	20.3	MCW0082	14.1	Saph1dawn	5.6	ADL0270	17.8
		LEI0117	4.0	MCW0247	80.7	ROS0018	11.4	PrlPromoter24bp		63.7	
		MCW0157	79.7	ADL0114	19.9	MCW0056	78.4	ADL0157			
3	9	MCW0083	85.6	MCW0139	15	ROS0001	37.7	ADL0327	20.6	LEI0118	28.8
		ADL0306	25.5	ADL0237	6.0	LEI0065	55.0	MCW0037			
4	7	ADL0317	81.9	ADL0145	23.8	LEI0122	14.3	ADL0246	47.1	LEI0094	59.0
		ADL0260	10.8	LEI0073							
5	5	MCW0090	38.6	ADL0292	25.4	LEI0145	22.2	MCW0032	27.5	ADL0166	

6	4	HUJ0008	14.7	ADL0138	3.4	ROS0070	4.1	ADL0323
7	4	ADL0279	15.4	ROS0019	4.1	ADL0180	39.0	ADL0169
8	4	MCW0275	20.6	MCW0305	18.9	MCW0100	63.6	ROS0075
9	4	ROS0078	41.4	ADL0191	18.5	MCW0135	55.0	ROS0030
10	2	ADL0209	15.8	ADL0231				
11	3	LEI0072	29.9	MCW0097	34.1	ADL0308		
12	1	ADL0044	0.1	ADL0044				
13	3	MCW0340	26.3	ADL0310	20.8	ADL0225		
14	2	MCW0123	21.2	ROS0005				
15	1	LEI0083	0.1	LEI0083				
17	2	ADL0199	0.0	ADL0202				

18	2	ADL0304	25.3	ROS0022		
23	2	ADL0289	89.5	LEI0090		
26	3	ADL0330	63.7	LEI0074	2.6	ADL0285
27	1	MCW0350	0.1	MCW0350		
28	1	ROS0085	0.1	ROS0085		
E22C19W28	1	ROS0054	0.1	ROS0054		
E47W24	1	LEI0080	0.1	LEI0080		
Z	2	MCW0258	0.2	LEI0075		

Chapter 4 Fine Mapping of Incubation (Broodiness) Trait

4.1 Introduction

The first step in QTL mapping is often to determine the general locations of QTLs using smaller numbers of markers (Abasht et al., 2006, Barton and Keightley, 2002, Hocking, 2005) and the second step is to focus on fine mapping of the regions containing the QTLs for traits like body fat, abdominal fat and bone traits to narrow down the confidence interval (Lionikas et al., 2010, Liu et al., 2008, Zhang et al., 2011a). There are two ways of fine mapping, to increase the numbers of polymorphic markers in the regions of the QTL or to sample more individuals to obtain the necessary recombinations. If markers are not very dense then the addition of more markers in the region of the QTL can be useful and if markers are already dense or the phenotype is relatively poorly measured then adding new individuals is better to get more recombinations and better estimate of phenotype (Brisbin and Peterson, 2007, Darvasi and Soller, 1997). These approaches help to limit the size of the genomic regions and therefore the number of positional candidate genes contained in the QTL region (Brisbin and Peterson, 2007, Darvasi and Soller, 1997, Nezer et al., 2003, Zhang et al., 2011b). This should make it easier to identify the gene or loci which are responsible for the effect and allow us to both understand the loci and find markers which are useful for selection. In context to the present study, there was a limitation in that the number of individuals cannot be increased and only alternative was to increase the number of markers in the region of QTLs.

Microsatellite markers are the regions of non-coding DNA containing short repeats. Each repeat motif is commonly two, three, or four base pairs in size, and the number of repeats is highly variable between different individuals (Georges, 2007). Single nucleotide polymorphisms (SNPs) markers are regions of the genome in which two

individuals differ by a single base pair (Slate et al., 2009). Microsatellite polymorphisms occur due to unequal crossing over or mutations extending or interrupting a series of repeats, whereas SNPs arise via point mutations (Syvänen, 2005, Xing et al., 2005). Because of the level of variation microsatellites have advantages, particularly they tend to be highly informative, they are however less abundant whilst SNPs are much more abundant in the genome. SNPs tend to be less informative but they are much easier to genotype in large numbers.

Fine-mapping involves the identification of markers that are very tightly linked to a targeted QTL or ideally a causative gene (Tarres et al., 2009). By using microsatellite markers high-resolution mapping would identify multiple closely linked QTLs that underlie each linkage or association peak (Ignal and Ilan, 2002).

Genetic maps therefore provide a powerful tool for gene identification, study, utilization and isolation.

The use of SNP markers across a QTL region offers the possibility of dense map coverage and is a shift towards genome wide association which is a powerful method for high resolution mapping (Anderson 2009) and has will facilitated the development of genome-wide SNP assays (de Koning and Hocking, 2007).

Single nucleotide polymorphisms (SNPs) are much more frequent than insertion or deletion of DNA or microsatellite repetitive regions and occur at high frequency in both non-coding regions and coding regions of the genome(Ignal and Ilan, 2002). Single nucleotide polymorphisms within coding regions showed either silent or synonymous polymorphisms and may have no effect on the protein coded by the gene or showed non- synonymous polymorphism which causing a change in a single amino acid in the protein sequence. The latter are have a greater chance of being the

functional polymorphisms that are responsible for phenotypic variation in traits (Slate et al., 2009). However, in many cases, the functional polymorphism responsible for variations in a trait may occur in intergenic regions (DNA sequences located between genes effecting expression or either quantity or location of the expression) or indeed some considerable distance from the gene affected as was the case for polydactyly long range enhancer described in the cross used here(Dunn et al., 2011).

In the present study, after using microsatellite markers, QTLs were further fine mapped by using SNPs markers to reduce the confidence intervals and also to move towards the ultimate identification of the actual mutation that causes the phenotypic variation in the two strains of chicken, White Leghorn and Silkie for incubation behaviour.

4.2 Objective

1. To test the hypothesis that genetic loci detected for incubation behaviour (Chapter 3) will be fine mapped by using SNP markers in F2 populations of chickens founded from White Leghorn and Silkie chickens.

2. To detect the possible QTLs, add SNP markers to chromosomes or linkage groups which have not been covered by the microsatellite map where possible.

4.3 Materials and Methods

4.3.1 Resource Population and Data Collection

Detail of resource population is described in chapter 2, section 2.2.and details of data collection of incubation status and early incubation was described in detail in chapter 3, section 3.3.3.

4.3.2 Genotyping

Blood samples were collected from all the individuals of F_0, F_1 and F_2. DNA was extracted by using the method described in chapter 2, section 2.5.1.

4.3.3 Strategy of dense genotyping

After initial analysis for potential QTLs, the number of markers was increased on chromosomes (2, 5, 7, 8, 9, 13, E22C19W28 and Z) and linkage groups which were not represented in the initial microsatellite screen were added (16, 19, 20, 21, 22, 24, 25, LGE64) by genotyping the entire population for 384 SNPs (Table 4-4).

In case of linkage groups 5, 7, 8, 9 and 13, selected SNPs were spaced at 2 cM distance. Markers on linkage group E22C19W28 were at a distance of 1cM as this is microchromosome with limited information and interesting loci so maximum SNP cover was adopted. Markers on Z chromosome were 5cM apart.

Regarding coverage of new linkage groups SNP markers were selected at a distance of 5cM apart on 16, 19, 20, 21, 22, 24, 25, LGE64,. But in the case of chromosome 20, markers were only 1cM apart because of importance of this chromosome in some of morphological traits QTL (Chapter 6).

4.3.4 Sources of SNPs selection

The SNP markers were selected from the Ensembl genome browser (http://www.ensembl.org/Gallus_gallus/Info/Index) using Biomart option for the White Leghorn and Silkie separately. SNP were selected further by comparing these with a list of validated SNPs (Groenen et al., 2009).

4.3.5 Golden gate Vera code genotyping assay

The SNP markers were genotyped by Golden gate Vera code genotyping assay (Illumina).

4.3.6 Goldon Gate Assay for Vera code

DNA activation is the first step in the GoldenGate Assay in which genomic DNA samples bind to paramagnetic particles. Next step is hybridization in which assay oligonucleotides, hybridization buffer, and paramagnetic particles are combined with the activated DNA. Three oligonucleotides are designed for each SNP locus. These are two allele-specific oligos (ASO) and third one is the locus-specific oligo (LSO). During the primer hybridization process, the ASOs and LSOs hybridize to the genomic DNA sample bound to paramagnetic particles. Extension of the appropriate ASO and ligation of the extended product to the LSO joins information about the genotype present at the SNP site to the address sequence on the LSO. Hybridization of the GoldenGate Assay products onto the VeraCode beads separates the assay products for individual SNP genotype readout. (http://www.illumine.com/technology/veracode_goldengate_assay.ilmn).

After hybridization, the BeadXpress® Reader is used for microbead code identification and fluorescent signal detection. Data was then analysed with Illumina's GenomeStudioTM data analysis software. (http://www.illumina.com/technology/veracode_goldengate_assay.ilmn).

Table 4-1 Results of SNP validation using Illumina Golden Gate Assay

Total number of SNP assays	**384**
Failed SNPs assay	0
Total number of successful SNP assays	384
No of monomorphic markers	163
No of polymorphic markers	221
Excluded SNPs due to missing genotypes among reference samples	03
Polymorphic Markers entered into resspecies	218

After completion of the assay, Bead Studio software was used to analyse SNP data and as a first pass to remove bad or un-informative markers. Out of 384 SNPs markers, 218 SNPs markers were informative and of suitable quality.

4.3.7 Next generation sequencing

After analysing these SNPs markers, an additional 4 SNPs were added in the peak region of the QTL on chromosome 5 including markers in gene coding regions. These SNPs markers were obtained by comparing the genome sequence of Silkie and White Leghorn produced using solexa (Illumina) next generation sequencing.

4.3.8 Map construction

After passing through the bead studio software, the SNPs were submitted to Res Species. Before this the genotype data were checked by genotype checker software (Paterson and Law, 2011) to find out if there were errors identifiable from the inheritance, this also identifies any bad individuals or markers which bead studio may not have highlighted. After individuals, markers or individual genotypes which were potentially poor quality or erroneous were masked, the remaining markers were then entered into resspecies database.

For map construction, both microsatellite and SNPs markers were exported from resspecies in the CRIMAP format. Marker order and map distances were estimated by using CRIMAP 2.4 software (Green et al., 1990) The CHROMPIC option was used to identify unlikely double crossovers. The FLIPS option with a 4-marker window was used to obtain the most likely order given the present data set. A sex-average linkage map was built by using the BUILD option. All markers used in this study were anchored into the chicken genome database (http://www.genome.uc-sc.edu/) to obtain the genome positions.

In case of chromosome 5 and 8 map length was very large. By using CHROMPIC option, it was possible to produce a map of sensible length for chromosome 5. For early incubation QTL on chromosome 8, map was constructed by interpolation of the new SNP markers using their physical position from the 2006 genome build and the genetic position of the microsatellite markers.

4.3.9 QTL Analysis

The BCF2 (Back cross F_2/ F_2 generation algorithm) analysis was carried out and implemented by using GridQTL software (Seaton et al., 2002). The QTL effects (additive and dominant effects) were fitted in a model with pen and family as fixed effects. The phenotypic variance explained by QTL was calculated as the difference in the residual sums of squares between the full model (including QTL) and reduced model (without QTL) (Liu et al., 2008). Significance thresholds were calculated by using permutation tests(Churchill and Doerge, 1994) . For each test point, a total of 1000 permutations were computed to determine the empirical distribution of the statistical test under the null hypothesis, and the 95% CI for each QTL was calculated from 1000 bootstrap samples (Visscher et al., 1996) .

4.4 Results

4.4.1 Summary statistics

Summary statistics of traits, incubation status and early incubation behaviour are described in detail in Chapter 3, section 3.4.2. Graphical presentation of summary statistics (Number of birds in each category) is mentioned in chapter 3, Figure, 3-1 and 3-2.

4.4.2 Significance of results

Initially, 80 microsatellite markers were genotyped on F_0, F_1 and F_2. After getting QTLs of incubation status and early incubation on chromosome 5 and 8, further screening was done with SNP markers on all chromosomes. 384 SNPs markers were

genotyped on whole population of F_0, F_1 and F_2. Out of these 218 SNPs markers were found polymorphic and used for the analysis.

Genome wide significant QTL and suggestive QTLs found for incubation status and early incubation behaviour in cross of White Leghorn (WLH) and Silkie (SLK) are presented in Table 4-1.

Additive and dominance effects of incubation status and early incubation behaviour traits QTLs in F2 cross of White Leghorn (WLH) and Silkie (SLK) are given in Table 4-2

Table 4-2 Genome wide significant QTL found for incubation status and early incubation behaviour traits in F2 population of White Leghorn and Silkie cross

Trait	Chromosome	F-ratio[1]	Flanking markers around Peak Position	Confidence Interval	Position (cM)
Incubation status	5	7.84*	snp_dio2_08-snp_tshr_06	75-123	100
	E22C19W28	6.01†	rs16687038 - rs16705784	0-26	13
	1	5.34†	LEI0146 - MCW0112	1-175	70
	18	5.51†	ADL0304	0-25	0
	19	5.93†	rs15846285 - rs15050199	0-16	1
Early Incubation	8	11.76**	rs16624982 - rs16625404	0-42	21
	26	6.07†	ADL0330	0-66	0
	1	6.16†	LEI0146 - MCW0112	3-100	66

[1]Significant at 0.05 (*) and 0.01(**) levels genome wide, and (†) suggestive

Table 4-3 Additive and dominance effects of Incubation status and early incubation behaviour traits in F2 population of White Leghorn and Silkie cross

Trait	Chromosome	Position (cM)	Additive ±S.E	Dominance ±S.E	Phenotypic Variance[3] (%)
Incubation status	5	100	0.075 ± 0.01	0.51 ± 0.12	8.97
	E22C19W28	13	-0.182 ± 0.09	0.46 ± 0.15	7.02
	1	70	0.23 ± 0.11	-0.63 ± 0.23	6.29
	18	0	-0.24 ± 0.09	0.32 ± 0.14	6.48
	19	1	-0.055 ± 0.01	-0.69 ± 0.20	6.25
Early Incubation	8	21	0.18 ± 0.039	0.10 ± 0.05	13.02
	26	0	-0.11 ± 0.04	0.11 ± 0.05	7.09
	1	66	0.06 ± 0.04	-0.36 ± 0.11	7.36

[3]Proportional decrease in the residual sums of squares by fitting the model with the QTL compared to the reduced model.

4.4.2.1 Incubation Status

The evidence for QTL affecting incubation status over the whole period (52 week) on chromosome 5 at 100cM was strong ($P<0.05$) (Table 4-2, Figure 4-1).

Initially there were five microsatellite markers on this chromosome (chapter 3). After the addition of 218 informative SNPs markers on all the chromosomes, marker density increased across the genome including chromosome 5 where number of markers increased to 35 (Table 4-4).

The 95% confidence interval (C.I) spanned a region around 45 cM (Table 4-1) having previously been 95 cM. The dominance effect of the QTL was 0.51 (Table 4-2).

The peak position of the QTL was initially between flanking markers LEI0145 and MCW0032 which after the addition of SNPs markers was resolved to between rs13587819 and MCW0032. Further screening of this region was made by the addition of 4 SNPs markers. These markers were obtained by direct comparing the sequence of White Leghorn and Silkie from next generation sequencing. Out of these four line specific markers, three were in the region of Dio2 gene and one in TSHR gene. After analysis of these line specific markers, it was found that peak position was close to the TSHR SNP and its physical position on chromosome 5 was around 43247848 (build 3.2).

Three other suggestive QTL for incubation status were found after the addition of SNPs markers. First a suggestive QTL was found on chromosome E22C19W28 at

13cM with F-value 6.01. The additive and dominant effect of this QTL is -0.18 and 0.46.

Another suggestive QTL was found on chromosome 1 with F-value 5.34 at 70cM. The additive and dominant effect of this QTL is 0.23 and -0.63. This QTL explains 6.09 % of the phenotypic variation (Table 4-3). The peak position of this QTL is in between LEI0146 - MCW0112.

The third suggestive QTL was found on chromosome 18 with F-value 5.51 at 0cM. Additive and dominant effect of the QTL is -0.24 and 0.32 respectively. This QTL explains 6.48 % of the phenotypic variance.

The final suggestive QTL for incubation status was found on chromosome 19 at 1 cM when all four of the QTL on chromosome 5, E22C19W28, 1 and 18 at 100cM, 13, 70cM and 0cM were fitted as background co-factors (Table 4-3). The peak position of this QTL is in between markers rs15846285 - rs15050199.

Figure 4-1 Plot of F-ratio versus relative QTL positions on chromosome 5 for incubation status (incubation behaviour recorded over 52 weeks). The relative position on the linkage map is presented on x-axis and the F-ratio on y-axis

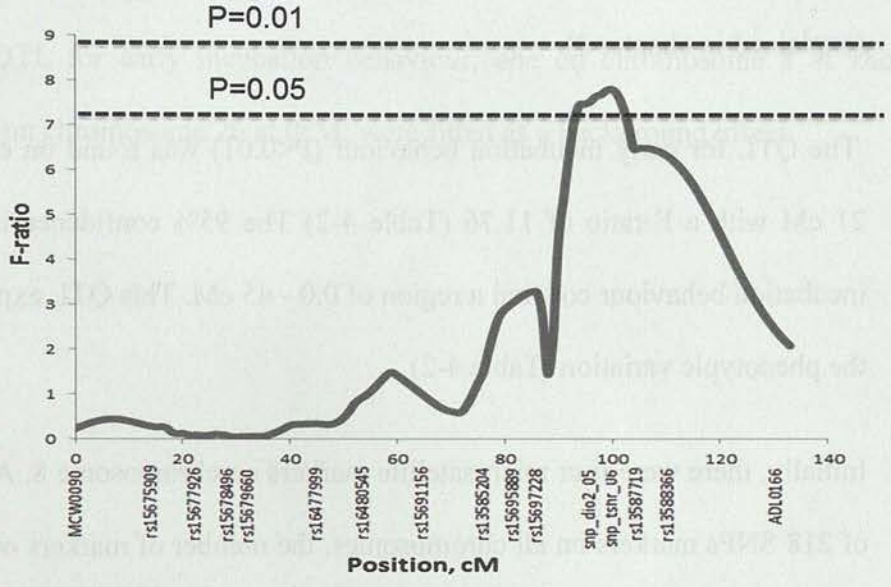
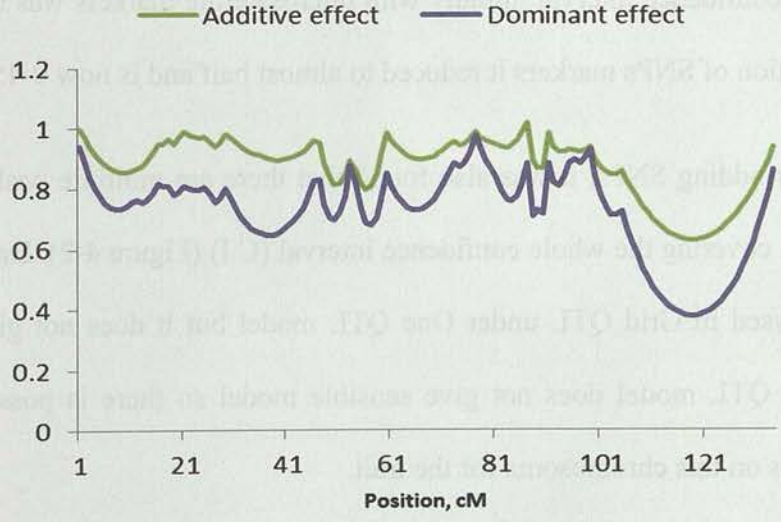


Figure 4-1a Graph showing additive and dominant effect of markers of on chromosome 5.



4.4.2.2 Early incubation behaviour

Using the data from the cumulative time periods a QTL was found which explained variance in the first period of 25-30 weeks age and this QTL was called 'Early incubation behaviour ' to distinguish it from the QTL observed over the total period (explained in chapter 3).

The QTL for early incubation behaviour ($P<0.01$) was found on chromosome 8 at 21 cM with a F ratio of 11.76 (Table 4-2) The 95% confidence interval for early incubation behaviour covered a region of 0.0 - 45 cM. This QTL explains 13.02 % of the phenotypic variation (Table 4-2).

Initially, there were four microsatellite markers on chromosome 8. After the addition of 218 SNPs markers on all chromosomes, the number of markers on chromosome 8 were increased to 50. The peak position of the QTL was initially in between MCW0275 - MCW0305. After adding SNPs, the peak position of QTL was between markers rs16624982 - rs16625404.

The confidence interval initially with microsatellite markers was 0-89.5 but with the addition of SNPs markers it reduced to almost half and is now 0-45cM.

After adding SNPs, it was also found that there are multiple peaks in the region of QTL covering the whole confidence interval (C.I) (Figure 4-2). Data for the trait was analysed in Grid QTL under One QTL model but it does not give a clear profile. Two QTL model does not give sensible model so there is possibility of multiple QTLs on this chromosome for the trait.

One suggestive QTL for early incubation behaviour was found on chromosome 26 at 0cM after the addition of 218 informative markers on all chromosomes, the additive and dominance effect of this QTL is -0.11 and 0.11 (Table 4-3).

Another suggestive QTL was found on chromosome 1 at 66cM, when both of above QTL for early incubation behaviour, one on chromosome 8 at 18cM and another on chromosome 26 at 0cM, were fitted as a background effect.

Figure 4-2 Plot of F-ratio versus relative QTL positions on chromosome 8 for Early incubation (25-30 weeks). The relative position on the linkage map is presented on x-axis and the F-ratio on y-axis

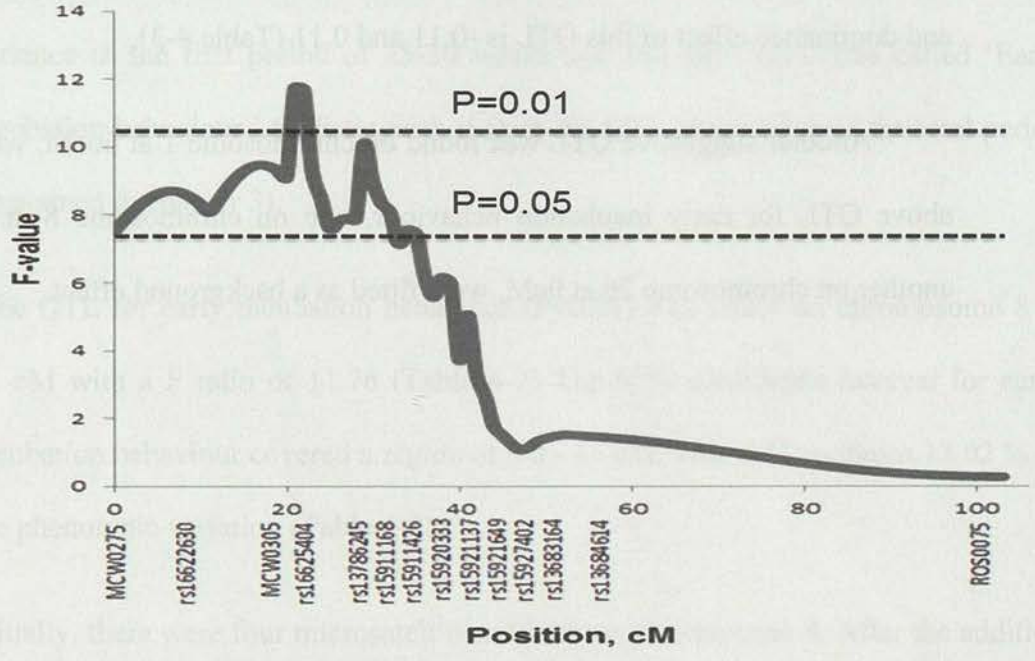
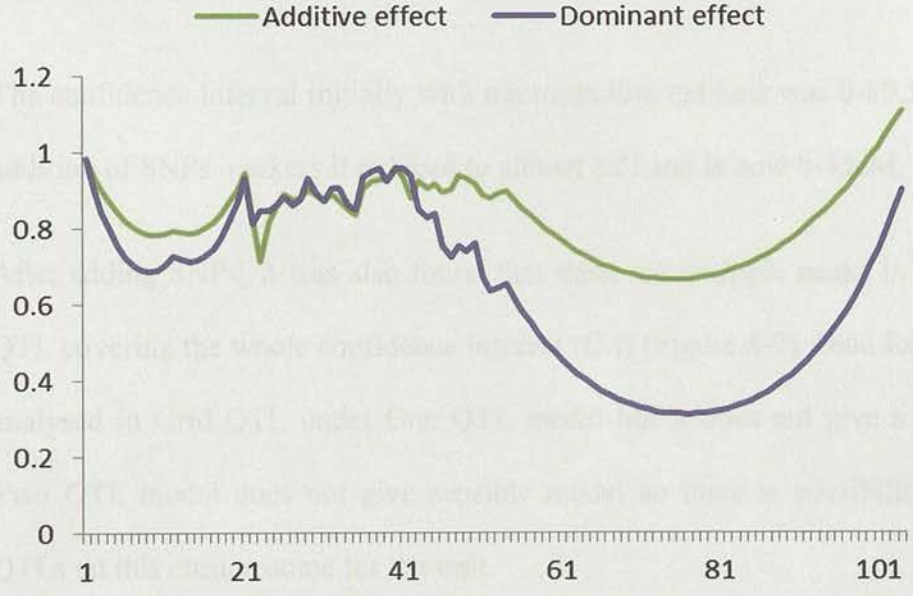


Figure 4-2a Graph showing additive and dominant effect of markers on chromosome 8



4.5 Discussion

Two significant QTL have been detected for the trait of incubation or maternal behaviour (one for incubation status and other for early incubation). The QTL for incubation status was found on chromosome 5 at 100cM was found significant at genome wide 5% level which cover period of 52 weeks. The targeted SNPs were used to refine the estimated position of the locus which in the case of the incubation status QTL on chromosome 5 was reduced to a 95% CI of 45 cM between flanking markers rs13587819 and MCW0032. The remaining QTL loci for incubation behaviour on chromosome 1, 18, 19 and E22C19W28 were suggestive

The QTL for early incubation behaviour on chromosome 8 at 21cM is significant at the genome wide 1% level. This QTL for the trait of early incubation behaviour is additive. After the addition of informative SNP markers in the region the QTL 95% CI is reduced from 89.5 to 45 cM. It was also found that there are multiple peaks in the region of QTL covering the whole confidence interval (C.I). Data for the trait was analysed in Grid QTL under One QTL model but it does not give a clear profile. The presence of 2 QTL at the loci was tested under two QTL model but was not supported. It remains possible that several linked QTL may exist at this locus for the trait. Other QTL for early incubation behaviour on chromosome 1 and 26 are suggestive.

The peak position of the QTL on chromosome 5 overlap with the location of the largest selective sweep observed in a study comparing domesticated breeds versus red jungle fowl (Rubin et al., 2010). Although the confidence interval is quite large but still there are number of genes that might play a major role in the reproductive

system of birds. These genes are TSHR, DIO2 and DIO3. The level of TSHR genes is increased in the pars tuberalis during the long photoperiod (Nakao et al., 2008b). This thyroid stimulating hormone receptor further stimulate and inhibit the activation of DIO2 and DIO3 respectively (Nakao et al., 2008b, Nakao et al., 2008a). This results in an increase in the conversion of T4 to the biologically active thyroid hormone T3 in the brain. It was also found that another thyroid hormone deiodinase (DIO1) is present in 95% CI of the second significant QTL on chromosome 8. These genes play a key role in the traditional thyroid hormone system controlling metabolic activity (Yoshimura, 2010).

The QTL for incubation status on chromosome 5 and 19 are over dominant. This means animals that have inherited DNA from the Silkie and White Leghorn at the loci are less and more likely to show incubation behaviour respectively. It is possible that the lines used in this cross, which have been separated for a considerable time, have been selected at the locus for fertility traits in separate environments resulting in independent accumulation of beneficial alleles that are not shared between lines. When combined, as in this cross, it showed heterosis which is often seen in line crosses (White et al., 1975). This can be the cause of most of over dominant QTL for the incubation behaviour which may be inversely correlated with beneficial reproductive traits. In the case of the loci on chromosome 5 it may be that both loci promote persistence and increased egg laying while the converse is true on chromosome 19.

The QTL on chromosome 1 for incubation status is dominant if we further dissect its effect it was found that Silkie allele promoting incubation behaviour and a dominant

WL allele acting to prevent it. Other remaining loci for incubation status on linkage group E22C19W28 and on chromosome 18 are dominant and the Silkie allele acts dominantly to reduce incubation behaviour.

In case of early incubation behaviour, the suggestive QTL on chromosome 1 is behaving in the same manner as in incubation status, the Silkie allele promoting incubation behaviour and a dominant WL allele acting to prevent it. The significant QTL for early incubation behaviour has an additive effect.

Hertwig and Schwarz (1934) used the same cross and found that the F1 have a high incidence of incubation behaviour, indeed in this study they surpass the level observed in the F_0 Silkie population. This illustrates that maternal behaviour is dominant. When this is taken in to account along with the mode of action of the individual loci and the physiology of the trait some potential reasons for the observations emerge. The combination of the high egg production of the White Leghorn strain and the propensity of the Silkie for maternal behaviour combined with low egg production may be the reason for this.

It is a necessary prerequisite that there is a period of exposure to oestrogen and progesterone from mature ovarian follicles for incubation behaviour to occur, as well as the correct environment including a nest with eggs(Sharp, 2009) . Therefore the longer a hen is in lay the greater the chance that the correct steroidal environment will coincide with the correct environmental stimuli to reinforce and propagate incubation behaviour.

This may explain why the loci on chromosome 19, 18 and E22C19W28 have WL alleles apparently promoting incubation behaviour. These loci may in fact be loci for increased egg production, a trait associated with White Leghorn, but not directly for incubation behaviour. This may be semantics, since as has been explained the onset of incubation behaviour is not compatible with high egg production .

A modification of that hypothesis would be that the loci on chromosome 5 may have importance both for production and fertility but also for the maintenance of incubation behaviour, possibly utilising common components in the basal hypothalamic TSH to T3 production pathway which includes TSHR and the deiodinase enzymes (Nakao et al., 2008a). It may be pertinent that thyroidectomy in a wild bird can remove the response of the reproductive axis to photoperiod that results in regression of the reproductive system on long days (Dawson et al., 2002). This was not perhaps what Romanov (Romanov et al., 2002) had in mind when he postulated two opposing loci but it offers an explanation of how a locus, which in naturally incubating populations of hens could not propagate, could increase in frequency in a population. The combination of the two alleles results in more eggs but also retains incubation behaviour. So heterozygotes have an advantage. Presumably when freed from the requirement for incubation of their own eggs, populations carrying the non-incubating allele could be strongly selected for, removing the restriction of incubation behaviour and increasing the persistency of egg production. In the White Leghorn this may have extended so that selection has reduced the drive for incubation behaviour to a minimal level whilst fertility is high.

Either way there seems to be a strong possibility that there is a balance between the length of time laying eggs and when egg laying is terminated by incubation behaviour occurring that has important implications for clutch survival in the wild and selection during domestication.

4.6 Conclusion

For the first time genetic loci that explain maternal behaviour have been described. However the mode of action of the QTL on chromosome 5, E22C19W28, 13, 18, 19 indicate the White Leghorn allele is promoting incubation behaviour or that heterozygotes have performance that exceeds the homozygotes. We believe some of these are due to changes in fertility which increases the chance of incubation behaviour. Therefore the QTL on chromosome 8 for early incubation behaviour is most likely to be a locus that has an effect directly on incubation since it is both for early incubation, where duration of reproductive activity is not important and the inheritance on chromosome 8 and chromosome 1 is as expected with the Silkie allele promoting incubation behaviour. Similarly the QTL on chromosome 1 has the correct mode of inheritance and may be directly for incubation behaviour. Because of the coincidence of the loci on chromosome 5 with the site of the strongest selective sweep in poultry, the TSHR, and the coincidence of the QTL on chromosome 1 and 8 with thyroid hormone activity QTL it would appear that the thyrotrophic axis may be critical to the loss of incubation behaviour or at least the selection for increased egg production.

Table 4-4 Map of SNPs markers and Microsatellite markers with genetic distance (cM) between markers on each linkage group used in the QTL analysis of the F2 population of White Leghorn and Silkie cross

Linkage group	No of Marker	Name of marker along with map distance(cM) between them on linkage group							
1	10	ROS0008	4.6	MCW0106	2.6	ADL0307	1.8	LEI0146	63.7
		MCW0112	18.3	LEI0071	17.8	LEI0101	22.4	ADL0314	83.5
		LEI0079	67.2	ROS0025					
2	13	LEI0163	1	MCW0205	20.3	MCW0082	14.1	Saph1dawn	5.6
		ADL0270	17.8	LEI0117	4	MCW0247	80.7	ROS0018	11.4
		PrlPromoter24bp	63.7	MCW0157	79.7	ADL0114	19.9	MCW0056	78.4
3	9	MCW0083	100	MCW0139	0.6	ROS0001	37.7	ADL0327	20.6
		LEI0118	28.8	ADL0306	25.5	ADL0237	6	LEI0065	55
		MCW0037							
4	7	ADL0317	81.9	ADL0145	23.8	LEI0122	14.3	ADL0246	47.1
		LEI0094	59	ADL0260	10.8	LEI0073			

5	35	MCW0090	10.6	rs15675732	4.8	rs15675909	2.3	rs15676095	3.9
		rs15677926	3	rs16475268	3.9	rs15678496	3.4	rs15679363	2.4
		rs15679660	5.4	rs16477739	5.5	rs16477999	5.5	rs15686197	3.3
		rs16480545	3.3	ADL0292	6.5	rs15691156	9.8	rs15693847	3
		rs13585204	0.1	rs13585301	4.3	rs15695889	0.1	LEI0145	0.1
		rs13585385	1	rs15696504	1.5	rs15697228	0.8	rs13585693	6.5
		rs13586162	2.5	rs13586348	1.4	rs13586520	3.8	snp_dio2_02	0.6
		snp_dio2_05	0.1	snp_dio2_08	0.8	snp_tshr_06	0.1	rs13587719	2.5
		MCW0032	6.2	rs13588366	29.6	ADL0166			
6	4	HUJ0008	14.7	ADL0138	3.4	ROS0070	4.1	ADL0323	
7	19	rs15824275	40.8	rs16579782	2	rs15838018	1.8	rs15839450	7.3
		rs15841467	2.6	rs13742691	5.1	rs13743076	5.7	rs15845829	0.8
		rs15846953	3.1	rs15848860	3.6	rs13780726	2.4	WNT10A	2.3
		WNT6_2	0.7	WNT6_4	5.3	ADL0279	8.1	rs13597183	8.5

		ROS0019	4.4	ADL0180	34.2	ADL0169			
8	47	MCW0275	11.16	rs16622630	9.44	MCW0305	1.29	rs16624982	0.46
		rs16625404	0.90	rs13663458	0.48	rs16626402	0.79	rs16627353	0.29
		rs13786245	0.39	rs15909994	0.25	rs15910144	2.59	rs15910206	0.28
		rs15910298	0.35	rs15910395	0.33	rs13679026	0.12	rs13679046	0.48
		rs16628438	0.78	rs15911168	0.51	rs15911426	0.24	rs15911612	0.72
		rs16629619	0.29	rs15912619	0.25	rs15912837	0.39	rs15913264	0.89
		rs15914383	1.33	rs15916143	0.97	rs16632921	0.62	rs16633712	0.07
		rs16633756	0.43	rs15919081	1.18	rs15919992	0.44	rs15920333	0.62
		rs13681019	0.17	MCW0100	0.17	rs15921137	0.41	rs15921649	0.32
		rs15922182	0.20	rs15922515	0.44	rs15923259	0.20	rs15923727	0.17
		rs15923952	0.65	rs15925157	0.21	rs15925447	0.48	rs15926175	0.42
		rs15926867	0.21	rs15927402	4.17	rs13683164	1.20	rs13683546	3.84
		rs13684614	50.51	ROS0075					

9	31	rs15943091	2.3	ROS0078	5.8	rs15943908	5.8	rs15945813	8.5
		rs16653557	13	rs15965754	9.6	rs15964255	5.2	ADL0191	10.3
		rs15963064	7	rs13766252	1.4	rs15959966	0.6	rs13765935	0.4
		rs13765728	0.6	rs15957961	1.6	rs16659946	7	rs15956208	7
		rs15952751	0	rs15951914	0.8	rs16654985	0.8	rs15948245	0.8
		rs13607034	0.8	rs13607257	0.8	MCW0135	7.1	rs13607828	15.8
		rs13608500	15.8	rs13801064	2	rs13795397	3.8	rs13735283	8
		rs13736302	2.5	rs13736402	3.6	ROS0030			
10	2	ADL0209	15.8	ADL0231					
11	3	LEI0072	29.9	MCW0097	34.1	ADL0308			
12	2	ADL0044	0.1	ADL0044					
13	30	MCW0340	3.6	rs15691830	3.6	rs14992784	12.6	rs13727671	2.3
		rs14990487	1	rs14990154	4.8	rs13727095	3.2	rs14989660	6
		rs15682386	4.5	rs15679637	2.2	rs15677407	20.6	rs13819338	20.6

		rs13788564	0	rs15696310	5.4	rs15697396	9.4	rs14996618	12.3
		ADL0310	5.7	rs14997641	29.6	rs14998128	27.7	rs15001532	0.9
		rs16036470	3.8	rs14998975	3.8	rs14999191	3.8	rs15000136	3.6
		ADL0225	7.3	rs15000293	7.3	rs15000594	0.6	rs15000720	13.3
		rs15000764	3.3	rs15000846					
14	2	MCW0123	21.2	ROS0005					
15	2	LEI0083	0.1	LEI0083					
17	2	ADL0199	5.1	ADL0202					
18	2	ADL0304	25.3	ROS0022					
19	4	rs15846285	8	rs15049662	8	rs15050199	2.6	rs15855444	
20	21	rs15173076	4.9	rs15173306	0.6	rs16166298	3.2	rs15174012	0.7
		rs16167068	1.1	rs13633117	0.8	rs15174716	2.5	rs16168078	1.5
		rs15175481	4.3	rs13633868	7.7	rs15176962	8.1	rs15177166	8
		rs15178122	8	rs13634772	2.4	rs15178545	1.1	rs15179004	1.1

		rs15179186	3.2	rs15179584	0.6	rs16009369	1.2	rs15179744	1.1
		rs13795178							
21	3	rs16180946	6.5	rs15184649	2.9	rs16719722			
22	7	rs15187286	3.1	rs16183708	5.7	rs16688617	5.7	rs15998289	43.8
		rs16078312	0	rs13820263	0	rs15187890			
23	2	ADL0289	89.5	LEI0090					
24	13	rs15209167	1.9	rs16193123	1.9	rs16193484	4.5	rs16194150	4.5
		rs15214714	4.5	rs15215963	12.6	rs16196741	1.6	rs16197289	3.9
		rs15222612	3.7	rs15227675	7.9	rs16198389	5.7	rs15225299	7
		rs16199759							
25	6	rs16070197	13.2	rs13740997	13.2	rs16734871	5	rs16687808	20.2
		rs16682513	8.2	rs16723881					
26	3	ADL0330	63.7	LEI0074	2.6	ADL0285			

27	2	MCW0350	0.1	MCW0350					
28	2	ROS0085	0.1	ROS0085					
E22C19W28	5	rs16686888 pMEL17	2.3	rs16687038	10.6	rs16705784	10.6	ROS0054	5.5
LGE64	1	rs16068205	0.1	rs16068205					
E47W24	2	LEI0080	0.1	LEI0080					

Chapter 5 Mapping of Morphological Traits

5.1 Introduction

A Mendelian trait is one that is controlled by a single locus and follows a simple Mendelian inheritance pattern. In such cases, a mutation in a single gene can cause a variation in the phenotype that is inherited according to Mendel's laws. In Mendelian inheritance, if offspring received a dominant allele from either parent they will have the dominant form of the trait. Only those that received the recessive allele from both parents present with the recessive phenotype. The recessive phenotype may theoretically not be observed in a number of generations, lying dormant in heterozygous individuals until one of the carriers produce offspring with another carrier of recessive allele and both pass it on to their child. Purely Mendelian traits are a tiny minority of all traits, since most phenotypic traits variation is due to several or many genes.

5.1.1 Importance of Morphological traits

Extensive phenotypic variation is a common feature among different breeds of chicken throughout the world, and some of the chicken breeds have been artificially selected for morphological traits (Wragg et al., 2012). In certain parts of the world, people pay higher prices for tasty meat and for the appearance of the birds (Fanatico and Born, 2002, Zhou, 2002). Producers adapt breeding strategies according to consumer demand and in some cases they need to pay attention to the bird appearance. In southern China and France, yellow skin and yellow shank or leg has been considered an indicator for a higher quality of meat and has symbolic importance for good luck (Yang and Jiang, 2005, Zanetti et al., 2010). For the live

market, consumers pay much more attention to the colour of the plumage, skin and shank as well as redness and size of comb. Black skin and shanks are widely accepted as symbolic of nutritive and tonic for the consumer beyond that delivered (Yang & Jiang 2005).

5.1.2 Chicken as a Model of Mendelian inheritance

The chicken was the first animal species in which Mendelian inheritance was studied (Siegel et al., 2006) and was also first among farm animals to have its genome sequenced. So the chicken has remained a key organism for genetic research (Siegel et al., 2006)..

Bateson's experiments with poultry offered the first demonstration of Mendelian heredity in the animal kingdom(Bateson, 1902). He crossed Indian games (pea comb), Dorking (rose comb) and Wyandottes (rose comb) with single combed White Leghorn fowls. From the F1 and F2 generations of crosses they found that pea comb and single comb both are dominant to rose comb from the 3:1 segregation of the trait in F2 population.

Dominant white colour was another one of the first traits to be investigated following Mendel's classical work (Bateson, 1902) and the mutation was assigned the gene symbol *I* for its inhibiting effect on pigmentation (Hurst, 1905). Dominant white is a breed characteristic of White Leghorns giving the birds a pure white plumage without any patterns or markings. (Kerje et al., 2004) found that dominant white colour is caused by a mutation in PMEL17, encoding a protein with a crucial function in the eumelanosome (Kerje et al., 2004).

Finding genetic loci for morphological traits is of great interest and in some cases also important from economic point of view. Finding genes for comb type is also a major consideration in the current study. Markers for this trait may be of interest to commercial breeding companies because due to cannibalism, birds can injure each other, especially on the comb and wattles (Millman, 2002). So it is of concern to the poultry breeding companies to minimize the comb size which may potentially enhance animal welfare.

Other morphological traits; crest type, feathered leg, feather type, fibromelanosis, skin colour, feather colour and pattern were also studied. In the current study of a cross of White Leghorn (WLH) and Silkie (SLK), all these traits are segregating in F2 population so it is possible to map the loci of these traits.

The genetic map for microsatellite markers was prepared with CRIMAP, although CRIMAP can be used to map the localisation of the putative trait locus on a map of markers. But in our case the exact inheritance of some of the trait like feathered leg was not known. This is the reason, the Grid QTL software was used for finding the genetic loci of the traits (Seaton et al., 2006) .

5.2 Objectives

To test the hypothesis that genetic loci controlling Mendelian traits will be detectable by mapping in an F_2 population founded from WL and Silkie chickens

5.3 Material and Methods

5.3.1 Resource Population

Detail of resource population is in Chapter 2, section 2.1.

5.3.2 Phenotype collection and categorization of trait

The potentially morphological traits: comb type, crest, feather type, feathered leg, skin colour, comb and wattle colour and spot, feather colour and pattern were recorded at the end of experiment. 318 birds were studied for the all of the Morphological traits except fibromelanosis. For the effect of fibromelanosis on the internal organs, 70 birds were killed at the end of experiment and their integument examined.

Initially data recorded for the phenotypic characteristics was in raw form. Recategorization of all of the traits was done to simplify the trait in a format suitable for analysis. These traits were appraised in the light of an updated literature review and codes were assigned to each category of the trait. This was helpful for the analysis of the trait and finally it helps to dissect the effect of the trait.

5.3.2.1 Morphological trait

1. Comb type single and rose comb
2. Crest type birds having crest, no crest
3. Feather type normal or Silkie
4. Ptilopody or Feathered leg birds having feathered legs or no feathered leg

For details of the codes for the categorisation of the traits see Table 5-1.

5.3.2.2 Comb and Wattle

1. Comb and wattle colour Red, Blackish red, Black
2. Comb and wattle spot Birds having spots or not

For details of the codes for the categorisation of the traits see Table 5-1.

5.3.2.3 Skin colour

1. Skin colour White, Grey and Black
2. Skin Spot Birds having spot or not

Each skin colour is further categorised on the basis of presence and absence of colour. For details of the codes for the categorisation of the traits see Table 5-1.

5.3.2.4 Fibromelanosis

Fibromelanosis influence the colour of the internal organs of the bird.

1. Connective tissue Meninges colour (colour of brain tissue)
2. Connective tissue Muscle colour (colour of muscle tissue)
3. Connective tissue Integument colour (colour of integument)
4. Connective tissue colour total (combined colour of above three traits)

There were three categories of colour, No Pigment, Patchy and Black (Table 5-1).

5.3.2.5 Feather colour

Feather colour and pattern of neck back and wing were studied and is categorized below

1. Feather colour birds having, white, off white, grey, light brown, brown or black colour

2. Father Pattern barred, laced, speckled or tipped

Each feather colour and pattern is further categorized on the basis of presence and absence of pattern and is explained in Table 5-1 along with codes.

Table 5-1 Categories and assigned codes of Morphological trait for the cross of White Leghorn X Silkie

Sr. No.	Trait	Categories	Code assigned
1	Comb type	Single Comb	1
		Rose Comb	2
2	Crest type	No crest	1
		Crest	2
3	Feather type	Normal	1
		Silkie	2
4	Feathered leg	Absent	0
		Present	1
5	Wattle/comb colour	Red	1
		Black	2
6	Wattle/comb spotting	No Spot	1
		Black Spots	2
7	Skin colour	White/Not white	1/0
		Grey/Not Grey	1/0
		Black/Not black	1/0
8	Skin spotting	No spot	1
		Black spot	2
9	Fibromelanosis	White/non white	1/0
	(Meningis, Muscles,	Black/non black	1/0
	Integument, Total)		
10	Feather Colour	White/Not white	1/0
	(Neck, Back, Wing)	Off white/Not off white	1/0

		Grey/Not Grey	1/0
		Light brown/Not Light browon	1/0
		Brown/not brown	1/0
		Black/Not black	1/0
11	Barred Feather	Not Barred	1
	(Neck, Back, Wing)	Barred	2
12	Laced feather	No lacing	1
	(Neck, Back, Wing)	Lacing	2
13	Specked Feather	Speckled	1
	(Neck, Back, Wing)	Not Specked	2
14	Leg colour	No dark leg	1
		Dark leg	2

5.3.2 Genotyping and Map construction

Genotyping and map construction detail are in Chapter 3 section 3.3.4 and 3.3.5.

5.3.3 QTL Analysis

Before QTL analysis, all of the phenotypic traits were assigned codes (Table 5-1) and data was entered into resSpecies (http://www.resspecies.org). A map of 80 microsatellite markers was used (Chapter 3, Table 3-3). For QTL analysis the genotype of 80 microsatellite markers and the phenotype file was exported from resSpecies and analysis was done by using Grid QTL software by a method as explained in Chapter 3, section 3.3.6.

5.4 Results

Due to the large phenotypic variation on between White Leghorn and Silkie, the current cross of both breeds provides a useful opportunity to find genetic loci for morphological traits. These traits including comb type, crest, feather type, feathered leg, skin colour and feather pattern were recorded at the end of the experiment. For the effect of fibromelanosis on the internal organs, 70 birds were killed at the end of experiment and their integument examined.

Genome wide significant genetic loci found for morphological traits in cross of White Leghorn (WLH) and Silkie (SLK) are presented in Table 5-2.

Additive and dominance effects of genetic loci of morphological traits in F2 cross of White Leghorn (WLH) and Silkie (SLK) at genome wide significant threshold (5%, 1%) are given in Table 5-2.

5.4.1 Comb type

Summary statistics for Comb type showed that out of 317 birds studied for comb type in the F2 generation, 72% birds had rose comb while 28% birds had single comb (Figure 5-1).

A significant QTL ($P<0.01$) for comb type was found on chromosome 7 with F ratio of 291.60 (Table 5-2, Figure 5-2). The 95% confidence interval (CI) for comb type ranged from 0.0 -2.0cM.

The peak position of QTL for comb type is on 0cM and is at the ADL0279 marker. The total number of microsatellite markers on chromosome 7 was 4.

Table 5-2 Genome wide significant QTL found for morphological traits in F2 population of White Leghorn and Silkie cross

Trait	Chromosome	F-ratio	Flanking markers around Peak Position	Confidence Interval	Position (cM)
Comb type	7	291.64**	ADL0279	0.0 – 2	0
Crest type	E22C19W28	102.76**	ROS0054	0.0 - 0.0	0
	2	5.1*	MCW0082- aph1dawn	1.0 – 339	41
Feathered leg	13	6.81*	ADL0310- ADL0225	0.0 – 47	47
	5	5.71*	MCW0032- ADL0166	18.5 – 113	113
	9	6.97*	ADL0191- ADL0191	22.0 - 78.5	47
Feather type	1	5.89*	ROS0008	0.0 – 290	0
	3	142.89**	LEI0118- ADL0306	166.0-172	169
Fibromelanosis (Connective Colour Integument)	17	7.96*	ADL0199	0.0 - 0.0	0
	26	5.54*	LEI0074- ADL0285	0.0 - 66.	66
	4	5.09*	LEI0094- ADL0285	14.5 – 236	232
Skin Colour	9	6.46*	ROS0078- ADL0191	4.0 – 99	13
Speckled Neck Feather	2	5.64*	PrlPromoter- MCW0157	0.0 – 306	186
	E22C19W28	16.81*	ROS0054	0.0 – 0	0

[1]Significant at 0.05 (*) and 0.01(**) levels genome wide, and ([†]) suggestive

Table 5-3 Additive and dominance effects of Morphological traits in F2 population of White Leghorn and Silkie cross

Trait	Chromosome	Position (cM)	Additive +S.E	Dominance +S.E	Phenotypic Variance[1]
Comb type	7	0	-0.44 ± 0.02	0.40 ± 0.03	65
Crest type	E22C19W28	0	-0.56 ± 0.04	0.10 ± 0.06	39
Feathered leg	2	41	0.15 ± 0.05	-0.06 ± 0.07	3
	13	47	-0.03 ± 0.03	-0.17 ± 0.05	4
	5	113	-0.06 ± 0.03	0.14 ± 0.05	4
	9	47	-0.06 ± 0.04	-0.19 ± 0.06	4
Feather type	1	0	0.12 ± 0.05	-0.13 ± 0.08	4
	3	169	-0.48 ± 0.03	-0.49 ± 0.05	47
Fibromelanosis	17	0	-0.43 ± 0.12	-0.40 ± 0.17	20
(Connective Colour Integument)	26	66	0.43 ± 0.15	-0.49 ± 0.19	15
	4	232	0.47 ± 0.16	-0.40 ± 0.22	14
Skin Colour	9	13	-0.07 ± 0.09	0.60 ± 0.17	4
Speckled Neck Feather	2	186	0.18 ± 0.06	0.15 ± 0.14	5
	E22C19W28	0	-0.18 ± 0.06	0.15 ± 0.14	10

5.4.2 Crest Type

318 birds of F2 population were studied for crest type. Out of these, 24 % birds had no crest, 76 % birds showed a crest. Summary statistics showed that this trait is controlled by an incomplete dominant gene (Figure 5-1).

Two genetic loci were found for crest type, one on chromosome 2 (P=0.05) and other significant (P<0.05) genetic locus was found on chromosome E22C19W28 (Table 5-2). The 95% Confidence Interval for crest type on chromosome 2 spanned a wide region of around 336cM (Table 5-2) while on chromosome E22C19W28 there was only one marker on the chromosome so the position was not known.

On chromosome 2 which is a large chromosome, there were 13 microsatellite markers whilst on linkage group E22C19W29, ROS0054 was the only marker.

5.4.3 Feathered leg

Summary statistics for feathered leg indicate that 77% birds showed this trait while 23 % birds showed no feathered leg (Figure 5-1).

Three putative QTL for feathered leg were found on chromosomes 5 (F = 5.71), 9 (F = 6.97) and 13 (F = 6.81) at 113cM, 47cM and 47cM respectively (Table 5-2). 95% confidence interval (CI) for these feathered leg QTL on chromosome 5, 9 and 13 were 18.5-113.8cM, 22.0-78.5cM and 0.0-47.0 cM respectively.

Number of microsatellite markers on chromosome 5 and 9 were 4 and on chromosome 13, there are 3 markers.

5.4.4 Feathered type

Summary statistics for feather type showed that out of 317 birds of F2 population studied, 256 birds showed normal feathering and 50 birds showed Silkie feathering (Figure 5-1).

One highly significant QTL ($P<0.01$) for feather type was observed on chromosome 3(Table 5-2, Figure 5-3) at 169cM while another QTL on chromosome 1 at 0cM approached significance ($P=0.05$) (Table 5-2). The length of C.I for a significant QTL on chromosome 3 was 6cM (Table 5-2).

5.4.5 Fibromelanosis

For this trait, only data on 70 birds were analysed. Out of these 70 birds, 62 % showed no pigmentation of connective tissue integument while 8 % birds showed patchy appearance and 30 % birds had black colour of integuments (Figure 5-1).

In the case of fibromelanosis, three genetic loci for connective tissue integument were found on chromosome 4, 17 and 26. The QTL on chromosome 4 at 232cM and on chromosome 26 at 66cM nearly approached significance ($P=0.05$) while the QTL for this trait on chromosome 17 at 0 cM was significant ($P<0.05$) but there was only one marker on this chromosome (Table 5-2). 95% C.I for QTL on chromosome 3 and 26 was (14.5-236.0cM) and (0.0 – 66.0 cM) respectively. The additive and dominance effect for the trait on chromosome 17 was 0.43 and 0.40 respectively and on chromosome 26 was 0.43 and 0.49 respectively while on chromosome 4 was 0.47 and 0.40 respectively (Table 5-3).

5.4.6 Skin colour

Out of 317 birds of the F2 population examined for skin colour, 57 % birds were of white colour, 12 % birds were of grey and 30 % birds had black colour (Figure 5-1).

The evidence for QTL affecting skin colour on chromosome 9 at 13cM was significant ($P<0.05$) but this QTL has a wide (4.0- 99.0) 95% confidence interval (Table 5-2).

The black colour is due to dermal melanin but is influenced by sex linked recessive gene *id* (Hutt 1949). In this study, no QTL appears on Z chromosome. It will require its location to be found and the animals carrying it to be removed to progress. At the moment the number of markers on the Z chromosome is small so no progress can be made.

5.4.7 Feather Pattern

Genetic loci for feather colour (back and neck), barred pattern (neck, wing and back) and speckled pattern (neck, wing and back) were found on chromosome E22C19W28. Only one marker on this chromosome was available.

Figure 5-1 Summary statistics of Comb type, Crest type, Feather type, Feathered leg, Fibromelanosis and skin colour of F2 birds in cross of White Leghorn and Silkie. Y-axis is showing the number of individuals in each category.

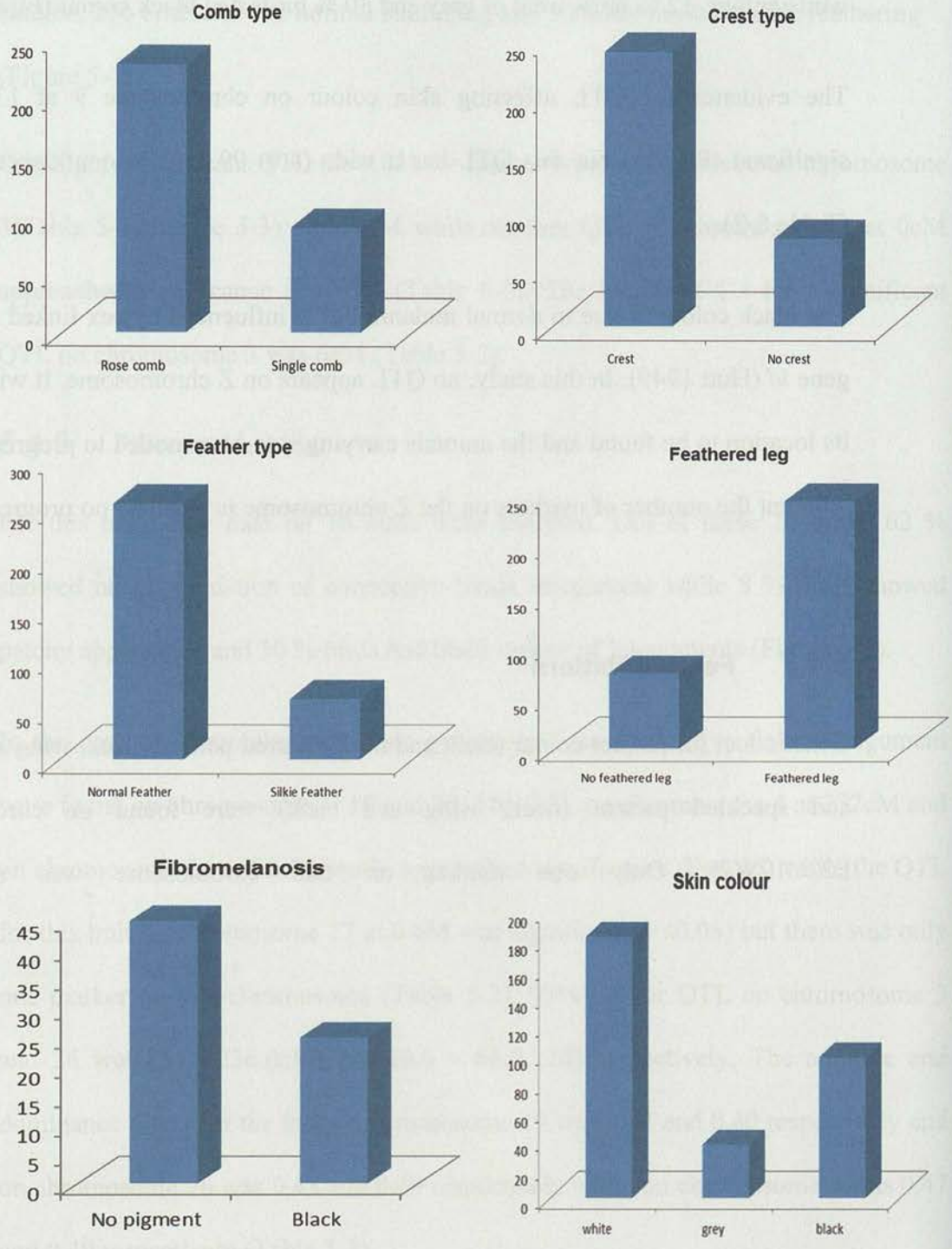
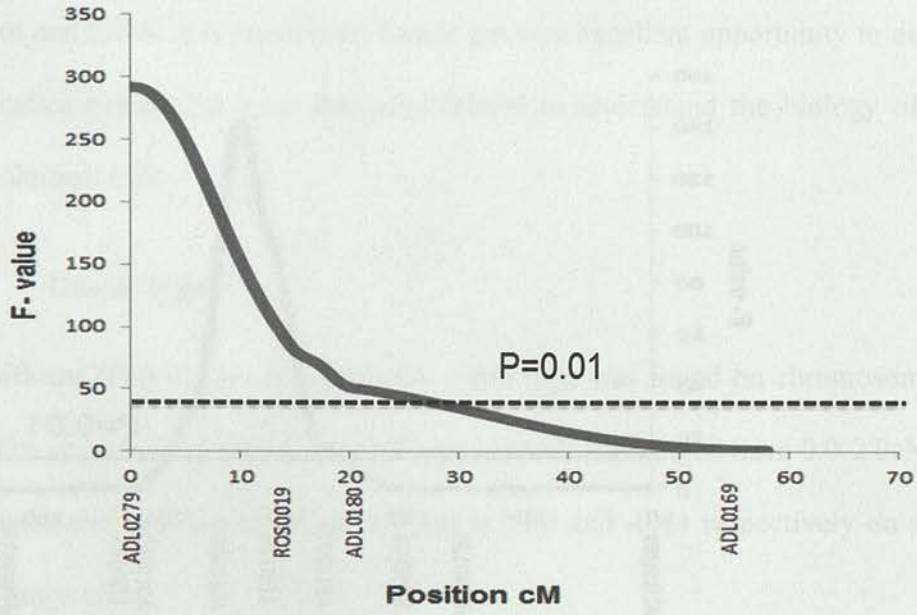


Figure 5-2 Plot of F-ratio versus relative QTL positions on chromosome 7 for Comb type. The relative position on linkage map is presented on x-axis and the F-ratio on y-axis



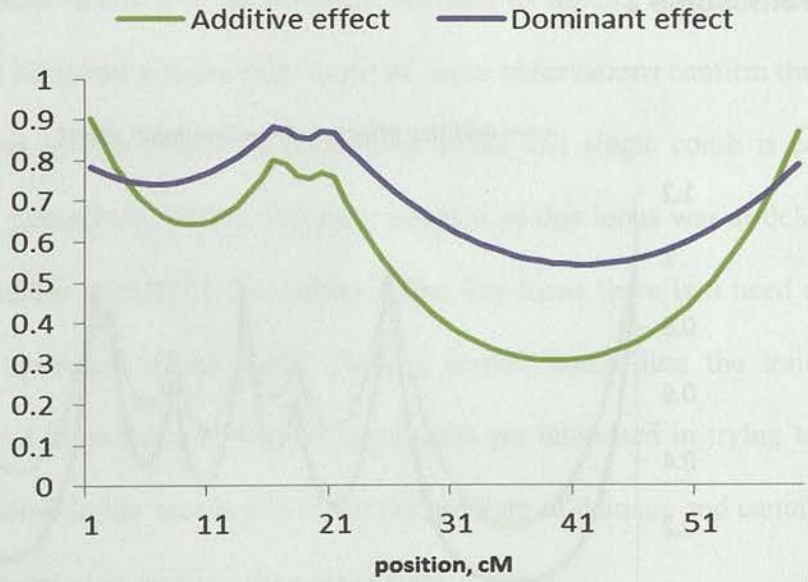


Figure 5-3 Plot of F-ratio versus relative QTL positions on chromosome 3 for Feather type. The relative position on linkage map is presented on x-axis and the F-ratio on y-axis

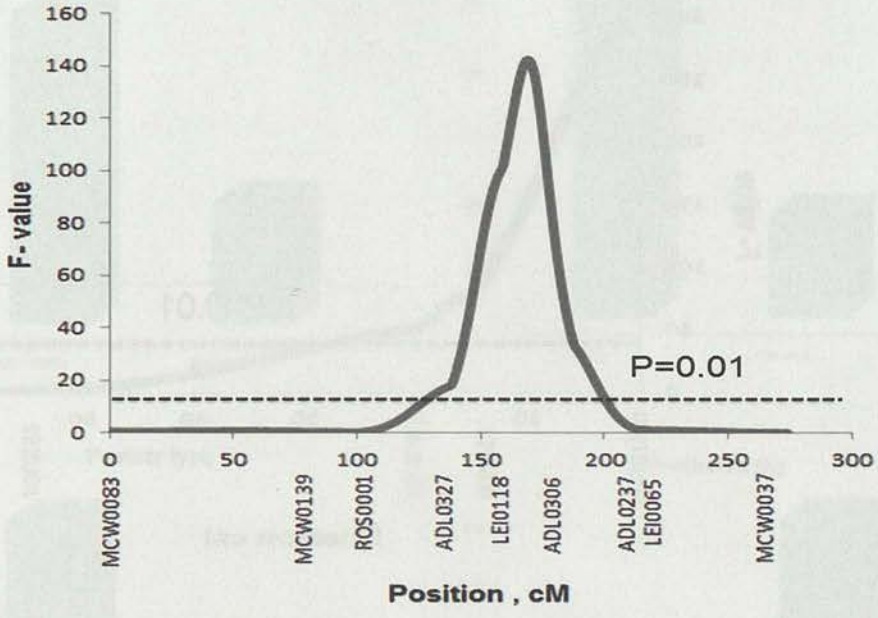
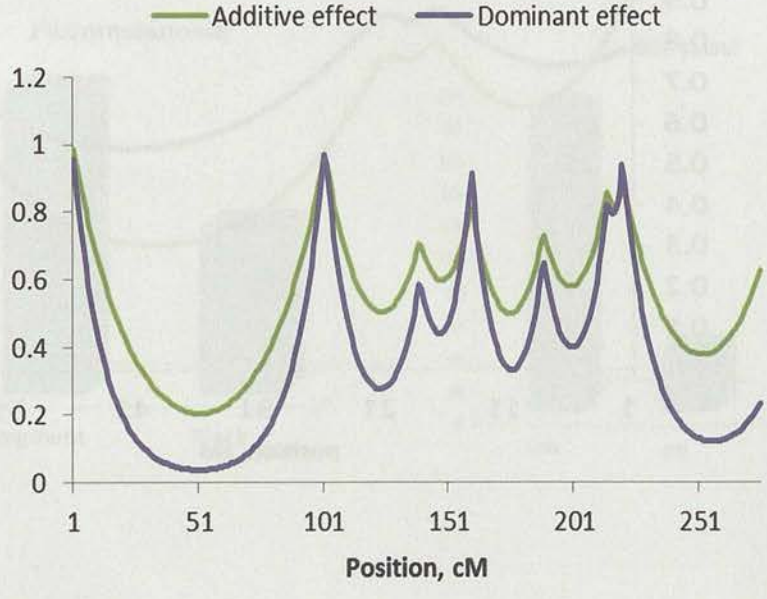


Figure 5-3a Graph showing additive and dominant effect of markers on chromosome 3



5.5 Discussion

Due to large difference in the morphological phenotypic characteristics of White Leghorn and Silkie, a cross of both breeds gave an excellent opportunity to discover the genetics behind the traits and also helpful to understand the biology of these morphological trait.

5.5.1 Comb type

A significant ($P<0.01$) genetic locus for comb type was found on chromosome 7 at 0cM. The confidence interval of comb type is small and ranged from 0.0-2.0cM. The dominance and additive effect of the trait is 0.40 and -0.44 respectively on a scale with a range of 1.

The significant value of additive effect also contributes towards wide variation of trait among both of these breed. It also indicates that increasing allele is coming from Silkie. These results with the summary statistics in which 72 % birds had rose comb and 28 % birds had single comb. Both of these observations confirm the inheritance of the trait as rose comb has dominance effect and single comb is controlled by recessive genes(Hutt, 1949). The peak position of this locus was at 0cM and which was at marker ADL0279. To further refine this locus there is a need to add more markers upstream of the locus. Finding marker controlling the trait is also of commercial importance as breeder companies are interested in trying to reduce the size the comb in the breeders to avoid the problem of damage and cannibalism. This trait is discussed in detail in the next chapter.

5.5.2 Crest type

One significant genetic locus for crest type was found on linkage group E22C9W28 and another one nearly approaching to (P= 0.05) was found on chromosome 2.

On chromosome 2, there were 13 microsatellite markers whilst on linkage group E22C19W29, ROS0054 was the only marker. The genetic locus for crest type on chromosome E22C19W28 is significant at 1% genome level. The dominant effect for the trait on the E22C19W28 is quite low but it has significant negative additive effect. It indicates that there is wide variation among both breeds for the trait. The negative value of additive effect indicates that increasing allele is coming from Silkie. Although currently there is only one marker on the linkage group but if this marker is further dissected it explains the effect match with the summary statistics.

To further dissect the trait there is need to map the region on both sides of ROS0054 then it should be possible to get a position and the confidence interval of the QTL.

5.5.3 Feathered Leg

Three putative genetic loci were found for feathered leg on chromosome 5, 9, and 13 with F- value 5.71, 6.97 and 6.81 respectively. Results of the current study are in agreement with finding of Somes (1992) who suggested that several loci are responsible for the Ptilopody (Pti loci), with varying modes of inheritance. But it was thought that Silkie feathered leg condition is thought to be caused by 1 or 2 incompletely dominant loci (Somes, 1992).

As summary statistics showed that this trait is controlled by dominant genes but it conflict with the finding as there are three genetic loci for the trait so cannot say surely about the mode of inheritance of all the genetic loci.

Earlier studies on feathered leg indicate that this trait is controlled by incompletely dominant genes (Danforth, 1929, Dunn and Jull, 1927a, Jull and Quinn, 1931). Dorshort et al. (2010) found a single genomic region on chromosome 13 is significantly associated with feathered leg and vulture hock phenotype.

5.5.4 Feathered type

One highly significant QTL ($P<0.01$) for feather type was observed on chromosome 3 at 169cM while another QTL on chromosome 1 at 0cM approached significance ($P=0.05$). The length of C. I for significant QTL on chromosome 3 was 6cM.

Summary statistics showed that out of 316 birds, 81 % birds had normal feathers while 19 % birds had Silkie feathers. These results indicated that Silkie feathering is controlled by homozygous recessive alleles.

5.5.5 Fibromelanosis and Skin Colour

In case of fibromelanosis, three QTL for connective tissue integument were found on chromosome 4, 17 and 26. QTL on chromosome 4 at 232cM and on chromosome 26 at 66cM nearly approached significance ($P=0.05$) while QTL for this trait on chromosome 17 at 0 cM was significant ($P<0.05$) but there is only one marker on this chromosome.

For this trait, strong dominant and additive effect was found for all three QTL indicating the contribution of the loci toward quantitative trait. For this trait, only data on 70 birds were analysed so there is need to study this trait further by increasing the number of birds. Out of these 70 birds, 62 % showed no pigmentation of connective tissue integument while 8 % birds showed patchy appearance and 30 % birds had black colour of integuments. Bateson and Punnet (1911) quoted by Hutt (1949) that black colour in internal organ is inhibited by a sex linked gene carried in brown leghorns. Dunn and Jull (1927) found a similar inhibitor in White Leghorns and concluded that it was either identical with the gene *Id*, inhibiting dermal melanin, or very closely linked with it. But in this study QTL for skin colour and fibromelanosis are present on different chromosomes, small number of birds studied for fibromelanosis and less number of markers on Z chromosomes might be the reason for such type of results.

The evidence for QTL affecting skin colour on chromosome 9 at 13cM was significant ($P<0.05$). Summary statistics showed that out of 317 birds of F2 population examined for skin colour, 57 % birds were of white colour, 12 % birds were of grey and 30 % birds had black colour. Black colour is due to dermal melanin but is influenced by sex linked recessive gene *id*, in homozygous (Hutt 1949). In this study, no QTL appears on the Z chromosome so it was not possible to take that in to account. It will require its location to be found and the animals carrying it to be removed. At the moment the number of markers on the Z chromosome is small. These traits will be discussed in detailed in next chapter.

5.5.6 Feather colour and Pattern

Genetic loci for feather colour (back and neck), barred pattern (neck, wing and back) and speckled pattern (neck, wing and back) were found on chromosome E22C19W28. Only one marker on this chromosome was studied.

Because the presence of a genetic locus for feather colour on linkage group E22C19W28 is likely to be due to presence of the dominant white locus on this chromosome (Kerje et al., 2004). To prove this hypothesis further SNPs marker along with microsatellite markers PMEL17 were added on this chromosome. The detailed discussion of this trait is in the next chapter.

5.6 Conclusion

Potential genetic loci for the Morphological traits (comb type, Crest type, feathered leg, feathered type, fibromelanosis and feathered pattern) were found. To further narrow down their confidence interval and to reach the genes controlling these traits, there is need to add further markers in the region of genetic loci for the trait.

Chapter 6 Fine Mapping of Morphological Traits

6.1 Introduction

As we discussed in the chapter 5, Morphological traits are economically important due to consumer preference for some traits like skin colour and feather colour whilst comb type may also have importance from a welfare point of view for the breeding companies who would like to reduce comb size to prevent damage to combs from inter bird aggression. Rose comb is smaller in size and less likely to be damaged. They are also of immense importance in fancy breeds of chicken and considered as breed characteristic in chickens and have the potential to inform us about events in development.

After the success of initial screening with microsatellite markers for important Morphological traits, further fine mapping was completed with SNP markers. The advantage of SNP markers in the region of QTL and their effect to limit the size of the genomic region where the underlying genetic difference resides is discussed in detail in chapter 4 section 4.1. Single nucleotide polymorphisms (SNP) are the most abundant class of DNA markers in the animal genome. With the advent of next-generation, high throughput, and cost-effective genotyping, the importance of SNP markers is an essential tool for molecular breeding (Groenen et al., 2009).

In the current chapter we target regions for fine mapping for most of the traits on the basis of their commercial usefulness. Further dissection of Crest is important as it potentially has a role in development of cerebral hernia (Wang et al., 2012). Feathered leg was also considered since finding regions and ultimately finding genes might shed light on scale to feather transition which would be novel and be of wider interest to biologists. A Feather type genetic locus was found on chromosome 3 with

a confidence interval of 3cM. This was not targeted for further fine mapping as we thought it had limited commercial value. In case of feathered leg genetic loci on chromosome 5 and 9, F values were reduced by the addition of SNPs markers on these chromosomes and were no longer significant.

Finding SNP as molecular markers for the traits will be helpful in Marker assisted selection and can be used in further breeding polices according to the preference, perhaps for introgression.

6.2 Objectives

1. To fine map the genetic loci controlling Morphological traits discovered in analysis of an F2 generation of cross of White Leghorn (WLH) and Silkie (SLK) chicken using a low density marker analysis by increasing the map density using SNP markers.

2. To increase the map coverage by including SNP markers from chromosomes not covered by the microsatellite map.

6.3 Material and Methods

6.3.1 6.3.1 Resource Population and Data Collection

The resource population is described in chapter 2, section 2.1.and details of phenotypic data collection of Morphological traits and categorisation of traits (comb type, crest type, feathered leg, feather type, fibromelanosis, skin colour, feather colour and pattern) are described in detail in chapter 5, section 5.3.1 and section 5.3.2.

6.3.2 6.3.2 Genotyping

Strategy of dense genotyping, sources of SNPs selection and method of SNP genotyping is described in detail in chapter 4, section 4.3.2.

With reference to current chapter, the regions focussed for fine mapping were the traits of comb type (chromosome 7), crest type (E22C19W28) and feathered leg (chromosome 13 and 9).

New linkage group added were chromosome 19, 20, 21, 22, 24, 25, and linkage group LGE64.

6.3.3 Next generation sequencing

After analysing genetic loci for the morphological traits, 3 additional SNPs were added in the peak region of the comb type QTL on chromosome 7 including markers in genes coding regions. These SNPs markers were obtained by comparing the genome sequence of Silkie and White Leghorn produced using solexa (Illumina) next generation sequencing.

6.3.4 Restriction Fragment Length Polymorphism

For the dissection of feather colour and pattern trait, a dominant white marker PMEL17 on linkage group was genotyped in the whole population of F0, F1 and F2 by using Restriction Fragment Length Polymorphism.

6.3.5 Map construction and QTL analysis

Detail of map construction and QTL analysis can be found in chapter 4 sections 4.3.7 and 4.3.8.

6.5 Results

6.5.1 Comb type

Initially, with low density markers (chapter 5, section 5.4.1) a significant genetic locus ($P<0.01$) for comb type was found on chromosome 7 with F ratio 291.60. Peak position was at 0cM coincident with the ADL0279 microsatellite marker. After initial screening with microsatellite markers, 15 SNPs were genotyped upstream of the ADL0279 marker and the data was reanalysed. A genome wide significant locus at 77cM ($P<0.01$) on chromosome 7 in the new map (Chapter 4, Table 4-4) with an increased F-value of 798 was found. The 95% confidence interval (C.I.) for the comb type locus was between 74-80 cM.

The additive and dominant effects of the comb type are -0.49 and 0.49. The trait has strong additive and dominant effect. The negative value of additive effect indicates that increased expression of the comb type phenotype is due to Silkie allele. Graphical representation of comb type QTL before and after the addition of SNP markers is given in Figure 6-1.

Inspection of the genome revealed 2 potential genes at or near the peak position which are known to be involved in several developmental process including regulation of cell fate and patterning during embryogenesis (Yates et al., 2005, van Amerongen and Nusse, 2009, Schubert et al., 2002). From the next generation sequencing, we got full sequence of White Leghorn and Silkie bird. Due to importance of these genes in the developmental process, Two SNPs in the coding region of WNT6 and one SNP in the coding region of WNT10A were obtained by

blasting the next generation sequences of both of these genes. These SNPs were genotyped in the entire population of F_0, F_1 and F_2. It was found that the peak position was between the SNP markers WNT10A and WNT6_2. The physical position of marker WNT10A is 23946588 Mb and position of marker WNT6_2 is 23962211 Mb on chromosome 7.

Table 6-1 Genome wide significant loci found for morphological traits in F2 population of White Leghorn and Silkie cross

Trait	Chromosome	F-ratio[1]	Flanking Markers around Peak Position	Confidence Interval (cM)	Position (cM)
Comb type	7	798**	WNT10A-WNT6_2	74-80	77
Crest type	E22C19W28	120.9**	rs16687038- rs16705784	4-17	7
Feathered leg	13	5.77*	rs15000136 - ADL0225	180-200	191
Skin colour white	20	16.17	rs15175481- rs13633868	55-59	56
Skin colour Grey	Z	6.69	rs16103438- rs13817193	81.5 - 344	108
Skin colour black	20	16.66	rs15175481- rs13633868	5.0 - 59	57
Leg colour	20	29.79**	rs15176962- rs15177166	53-62	60
Skin spot	20	9.12**	rs15174012- rs16167068	12-61	59
Comb colour	20	41.04**	rs15179004-rs15179186	55-59	57
Fibromelanosis (Integument)	20	7.56*	rs15176962- rs15177166	12-60	57
Meninges	20	10.4*	rs15176962- rs15177166	20-60	57
Muscle membranes	20	9.81*	rs16167068- rs13633117	10-60	28

[1]Significant at 0.05 (*) and 0.01(**) levels genome wide, and (†) suggestive

Table 6-1 continued Genome wide significant loci found for morphological traits in F2 population of White Leghorn and Silkie cross

Trait	Category	Chromosome	F-ratio[1]	Flanking Markers around Peak position	Confidence Interval (cM)	Position (cM)
Feather colour Neck	White	Z	7.32	rs13793820- rs13768797	174.0 - 490.0	321cM
	Of white	E22C19W28	27.61	ROS0054- PMEL17	12.5 - 29.0	26cM
	Grey	E22C19W28	21.54	rs16705784-ROS0054	0.0 - 27.0	18cM
	Light brown	4	6.14	ADL0246- LEI0094	0.0 - 236.0	142cM
	Black	E22C19W28	52.29	ROS0054- PMEL17	26.0 - 29.0	29cM
Feather colour wing	white	Z	6.67	rs13793820- rs13768797	174.0 - 490	320cM
	Off white	E22C19W28	30.18	ROS0054- PMEL17	16.0 - 29.0	24cM
	Black	E22C19W28	46.56	ROS0054- PMEL17	26.0 - 29.0	29cM
	Light brown	4	10.18	ADL0246- LEI0094	92.0 - 162.0	146cM
Feather colour back	White	Z	8.04	rs13793820- rs13768797	174-490	321cM
	Off white	E22C19W28	28.53	ROS0054- PMEL17	15-29	27cM

		Z	7.41	rs13793820- rs13768797	56-136	324cM
	Black	E22C19W28	48.64	ROS0054- PMEL17	26-29	29cM
	Light brown	4	10.18	ADL0246- LEI0094	92-160	146cM
Barring	Neck	E22C19W28	26.19**	rs16705784-ROS0054	13-28	25
	Wing	E22C19W28	25.31**	rs16705784-ROS0054	16-28	25
Lacing	Neck	E22C19W28	22.18**	rs16705784-ROS0054	0-29	27
	Wing	E22C19W28	19.16**	rs16705784-ROS0054	0-29	27
	Back	E22C19W28	19.75**	rs16705784-ROS0054	0-27	23
Speckled	Neck	E22C19W28	13.77**	rs16705784-ROS0054	18-29	28
	Wing	E22C19W28	26.63**	rs16705784-ROS0054	18-29	29
	Back	E22C19W28	17.19**	rs16705784-ROS0054	19-29	28

[1]Significant at 0.05 (*) and 0.01(**) levels genome wide, and ([†]) suggestive

Table 6-2 Additive and Dominance effect of Morphological traits in F2 population of White Leghorn and Silkie cross

Trait	Chromosome	Position (cM)	Additive ±S.E	Dominance ±S.E	Phenotypic Variance[1] (%)
Comb type	7	100	- 0.49 ± 0.01	0.49 ± 0.02	86
Crest	E22C19W29	7	-0.44 ± 0.03	0.39 ± 0.08	50
Feather leg	13	191	0.04 ± 0.03	-0.18 ± 0.06	5
Skin colour White	20	56	-0.17 ± 0.07	0.24 ± 0.05	13
Skin colour Grey	Z	108	-0.04 ± 0.02	0.50 ± 0.13	5
Skin colour black	20	57	0.09 ± 0.06	-0.26 ± 0.04	12
Leg colour	20	60	0.21 ± 0.03	-0.16 ± 0.05	19
Skin Spot	20	59	-0.12 ± 0.04	0.17 + 0.06	13
Comb colour	20	57	-0.50 ± 0.07	0.01 ±0.09	25
Fibromelanosis (Integument)	20	57	-0.23 + 0.09	0.22 + 0,12	22
Meninges	20	57	-0.62 ± 0.15	0.29 ± 0.21	27
Muscles	20	28	-0.40 ± 0.14	0.26 ± 0.20	27

[3]Proportional decrease in the residual sums of squares by fitting the model with the QTL compared to the reduced model.

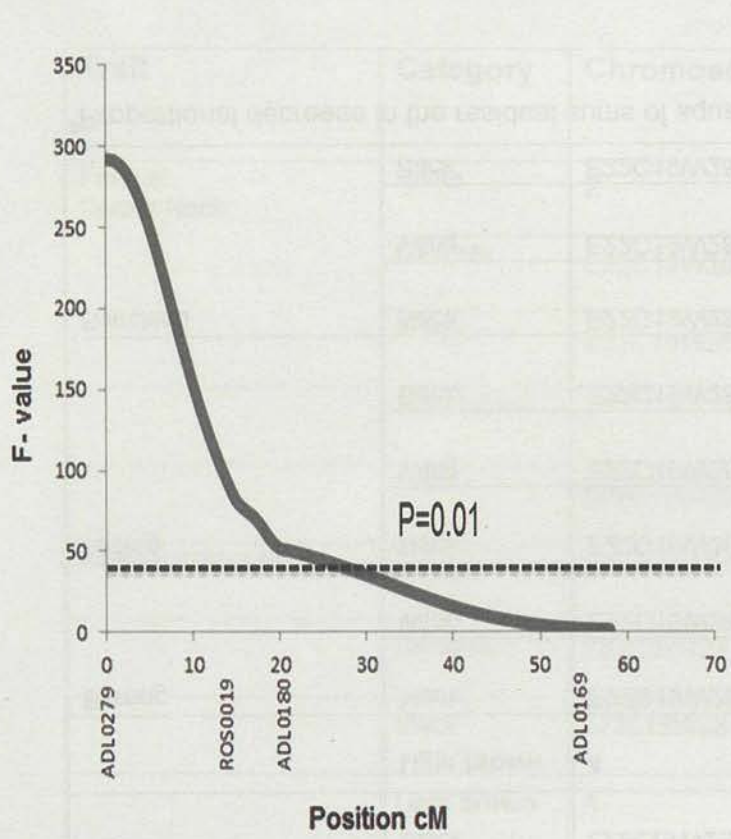
Table 6-2 continued Additive and Dominance effect of Morphological traits in F2 population of White Leghorn and Silkie cross

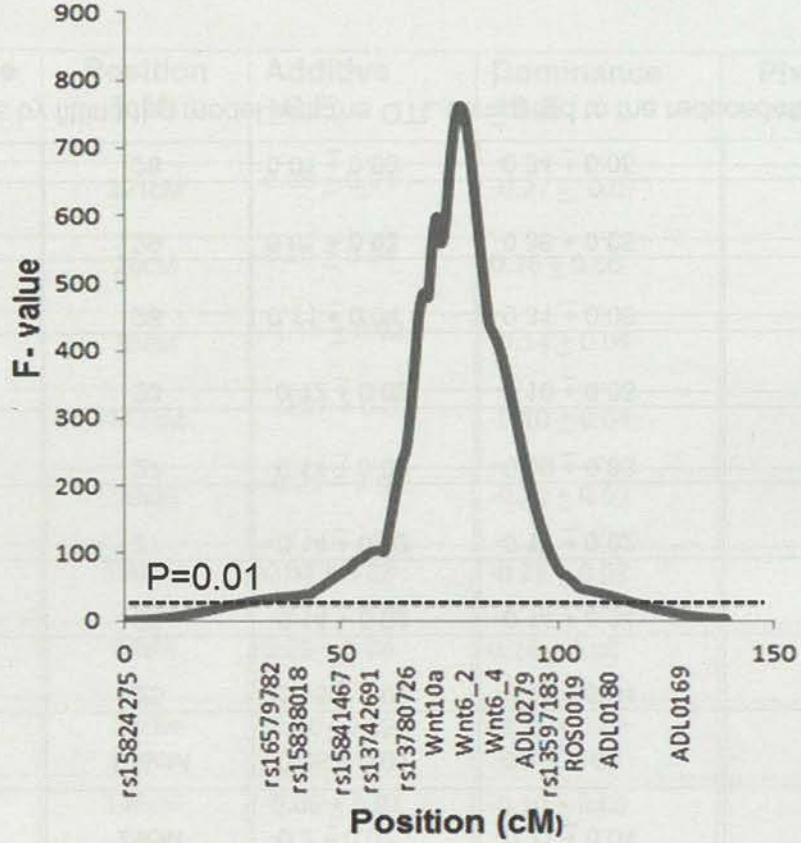
Trait	Category	Chromosome	Position (cM)	Additive ±S.E	Dominance ±S.E	Phenotypic variation[3] (%)
Feather Colour Neck	White	Z	321cM	0.05 ± 0.01	-0.27 ± 0.07	6
	Of white	E22C19W28	26cM	0.25 ± 0.04	0.26 ± 0.06	19
	Grey	E22C19W28	18cM	-1.15 ± 0.02	-0.14 ± 0.04	15
	Light brown	4	142cM	-0.07 + 0.02	-0.10 ± 0.04	5
	Black	E22C19W28	29cM	-0.21 + 0.02	-0.22 ± 0.03	30
Feather colour wing	white	Z	320cM	0.04 ± 0.02	-0.25 ± 0.07	6
	Off white	E22C19W28	24cM	0.26 ± 0.04	0.26 ± 0.06	20
	Black	E22C19W28	29cM	-0.20± 0.02	-0.21 ± 0.03	28
	Light brown	4	146cM	-0.06 ± 0.01	-0.10 ± 0.03	8
Feather colour back	White	Z	321cM	0.03 ± 0.01	-0.28 ± 0.07	6

	Off white	E22C19W28	27cM	0.24 ± 0.04	0.31 ± 0.06	19
		Z	324cM	-0.04 ± 0.02	0.30 ± 0.02	6
	Black	E22C19W28	29cM	-0.2 ± 0.02	-0.21 ± 0.04	29
	Light brown	4	146cM	-0.06 ± 0.01	-0.1 ± 0.03	8
Barring	Neck	E22C19W28	25	-0.15 ± 0.02	-0.15 + 0.04	18
	Wing	E22C19W28	25	-0.14 ± 0.02	-0.14 ± 0.04	17
Lacing	Neck	E22C19W28	27	-0.14 ± 0.02	-0.12 ± 0.02	16
	Wing	E22C19W28	27	-0.11 ± 0.02	-0.09 ± 0.03	13
	Back	E22C19W28	23	-0.12 ± 0.02	-0.10 ± 0.03	14
Speckled	Neck	E22C19W28	28	0.11 ± 0.04	-0.31 ± 0.05	10
	Wing	E22C19W28	29	0.01 + 0.03	-0.36 + 0.05	15
	Back	E22C19W28	28	0.01 ± 0.03	-0.34 ± 0.05	13

[3]Proportional decrease in the residual sums of squares by fitting the model with the QTL compared to the reduced model

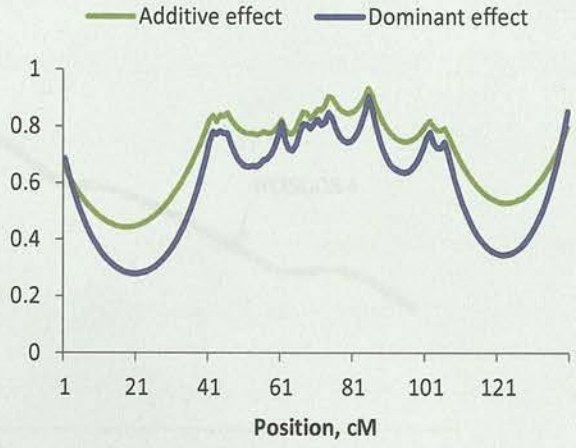
Figure 6-1 Plot of F-ratio versus relative QTL positions on chromosome 7 for comb type before and after fine mapping. The relative position on the linkage map is presented on x-axis and the F-ratio on y-axis





QTL of comb type before Fine Mapping	QTL of comb type after Fine Mapping

Figure 6-1a Graph showing additive and dominant effect of markers on chromosome 7.



6.5.2 Crest

A highly significant locus (P<0.01) for Crest was found on linkage group E22C19W28 at 7cM with an F-value of 120.9 which spanned a region of around 13cM (Table 6-1).

The additive and dominant effect of crest type is -0.44 and 0.39 (Table 6-2). The negative value of additive effect indicates that increased expression of the crest phenotype is due to Silkie allele. The peak position of the locus was in between markers rs16687038 and rs16705784.

Initially, only one microsatellite marker, ROS0054, was mapped on this chromosome giving a peak position of the locus at 0cM (chapter 5, section 5.4.2). In the case of

fine mapping, four SNPs markers and one microsatellite marker were added to this chromosome, refining the peak position at 7cM.

Figure 6-2 Plot of F-ratio versus relative QTL positions on Linkage group E22C19W28 for Crest type before and after fine mapping. The relative position on the linkage map is presented on x-axis and the F-ratio on y-axis. Initially (Chapter 5), ROS0054 was the only microsatellite marker that was mapped on E22C19W28.

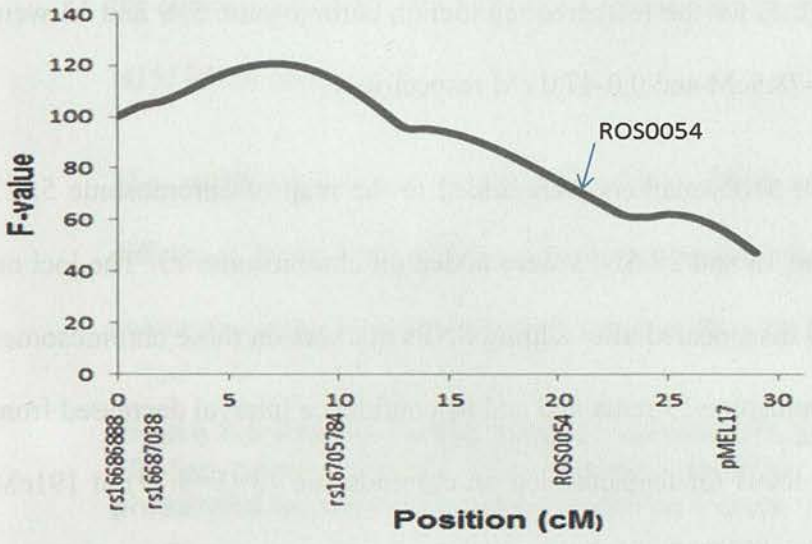
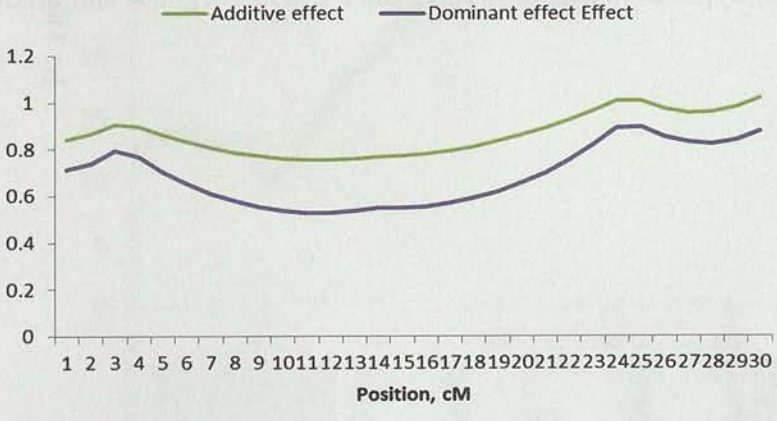


Figure 6-2a Graph showing additive and dominant effect of markers on linkage group E22C19W28.



6.5.3 Feathered leg

Initially, when data was analysed with microsatellite markers, three putative loci for feathered leg were found on chromosomes 5 (F = 5.71), 9 (F = 6.97) and 13 (F = 6.81) at 113cM, 47cM. and 47cM respectively (Chapter 5, Table 5-2).The 95% confidence interval (C.I) for the feathered leg loci on chromosome 5, 9 and 13 were 18.5-113.8cM, 22.0-78.5cM and 0.0-47.0 cM respectively.

For fine mapping, 30 SNPs markers were added to the map of chromosome 5, 27 SNPs on chromosome 13 and 28 SNPs were added on chromosome 13. The loci on chromosome 5 and 9 disappeared after adding SNPs markers on these chromosomes but the locus on chromosome 13 remained and its confidence interval decreased from 47cM to 20cM. The locus for feathered leg on chromosome 13 (F=5.77) at 191cM had a 95% C.I between 180-200 cM (Table 6-1).

The additive and dominant effect was 0.04 and -0.18 (Table 6-2). The locus has significant dominant effect showing the deviation of the heterozygous animals from the mean of the two types of homozygous animals i. e White leghorn and Silkie.

6.5.4 Comb colour

After adding SNP markers on all chromosomes, a highly significant (P<0.01) locus for comb colour was found on chromosome 20 with an F-value of 41.04. The 95% C.I was between 55 and 59cM. The length of the C. I. is very small suggesting that the genes controlling the trait are between the flanking markers rs15179004 and rs15179186 (Table 6-2).

The comb colour has significant additive effect of -0.50 indicating the breed difference for the trait. The negative value denotes the increase expression of comb colour phenotype is due to Silkie allele rather than the White leghorn allele.

Figure 6-3 Plot of F-ratio versus relative QTL positions on chromosome 20 for comb colour. The relative position on the linkage map is presented on x-axis and the F-ratio on y-axis

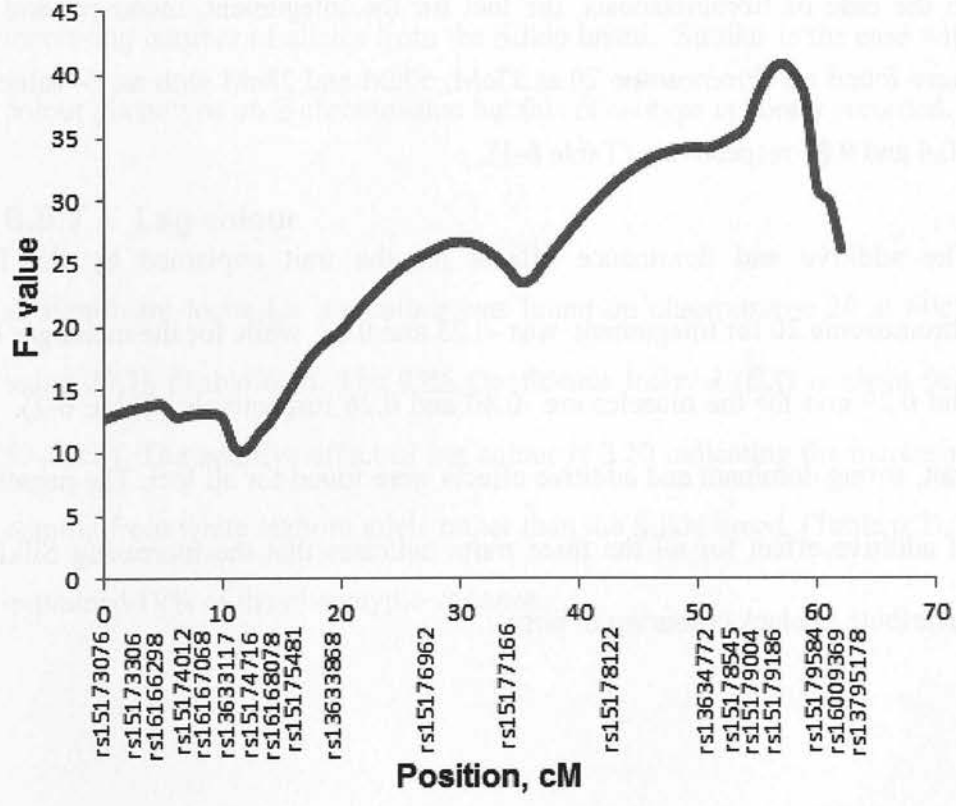
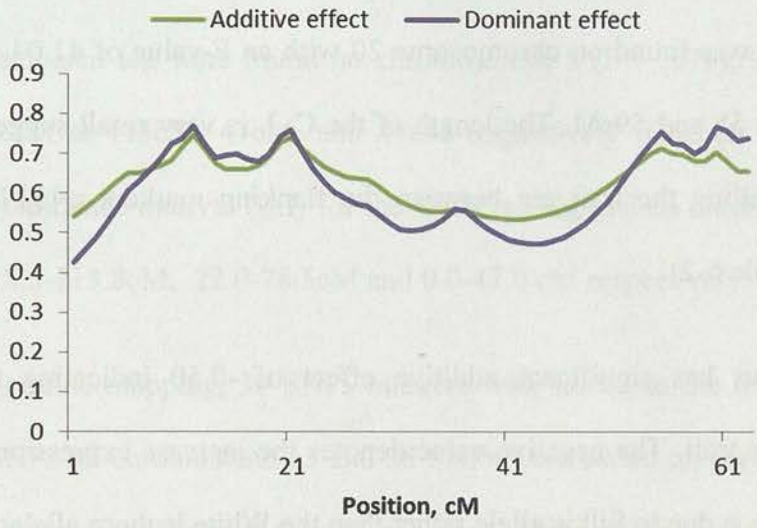


Figure 6-3a Graph showing additive and dominant effect of markers of on chromosome 20



6.5.5 Fibromelanosis

In the case of fibromelanosis, the loci for the integument, meninges and muscles were found on chromosome 20 at 57cM, 57cM and 28cM with an F- value of 7.56, 10.4 and 9.81 respectively (Table 6-1).

The additive and dominance effects for the trait explained by the locus on chromosome 20 for integument was -0.23 and 0.22 while for the meninges are -0.62 and 0.29 and for the muscles are -0.40 and 0.26 respectively (Table 6-2). For this trait, strong dominant and additive effects were found for all loci. The negative value of additive effect for all the three traits indicates that the increasing Silkie alleles contribute to black colouring in birds.

6.5.6 Skin colour

Out of 317 birds of F2 population examined for skin colour, 57 % birds were of white colour, 12 % birds were of grey and 30 % birds had black colour.

The evidence for locus affecting white and black skin colour on chromosome 20 at 56cM and 57cM were significant ($P<0.01$) and these loci having 95% confidence interval range of 55-59 cM and 5-59cM respectively(Table 6-1). A locus affecting white and grey skin colour was found on chromosome Z at 108cM with an F-value of 6.69.

The additive and dominant effect of the skin colour is given in Table 6-2. The negative value of loci for white and black phenotype on chromosome 20 was negative, indicating that proportion of white and black animals decrease with increasing number of alleles from the Silkie breed. Similar is the case with the grey colour phenotype on Z chromosome but this phenotype is poorly recorded.

6.5.7 Leg colour

A significant locus for leg colour was found on chromosome 20 at 60cM with F-value 29.79 (Table 6-1). The 95% Confidence interval (C.I) is about 9cM ranging 53-62cM. The additive effect of leg colour is 0.20 indicating the increasing allele is coming from white leghorn allele rather than the Silkie breed. (Table 6.2). This locus explained 19% of the phenotypic variance.

6.5.8 Feather colour

The highly significant loci for off white and black feather colour in the region of wing were found on chromosome E22C19W28 at 24cM and 29cM with F-value 30.18 and 46.56 respectively (Table 6-1). But a white wing feather colour locus was found on Z chromosome at 320 cM with an F-value of 6.67. Light brown wing feather colour locus was found on chromosome 4 at 146cM. The 95% CI for the off white and black feather colour in wing region ranged from 16-29cM and 26-29cM respectively (Table 6-2).

The loci for the white and off white feather colour in the region of back were found on chromosome Z at 321cM and 324cM with an F value of 8.04 and 7.41. while the other QTL of off white colour is found on linkage group E22C19W28 at 27cM with an F value of 28.53. Other locus found on the same linkage group is of black feather colour at 29cM with F-value of 48.94. Light brown feather colour was found on chromosome 4 at 4 at 146cM with an F-value of 10.18(Table 6.1). The 95% C.I of off white and black feather colour on E22C19W28 linkage group ranged from 15-29cM and 26-29cM respectively while the 95% C.I of white and off white feather colour in the region of back ranged from 174-490cM and 56-136cM respectively.

The highly significant QTLs for off white, grey and black neck feather colours were found on linkage group E22C19W28 at 26cM, 18cM and 29cM respectively with the F.-value of 27.61, 51.54 and 52.29 respectively (Table 6.1). While the loci for the white colour and light brown feathers were found on chromosome Z and 4 with an F-value of 7.32 and 6.14 respectively (Table 6.1).

The additive and dominant effects of all the feather colour loci in the region of wing, neck and back region are given in Table 6.2. The white feather colour in all the regions have stronger dominant effect than the additive effect and denote the deviation of heterozygotes from the mean of two of the parents, White leghorn and Silkie. The positive value of dominant and additive effect of off white feather colour in all the regions indicate that White leghorn allele would results in higher proportion of off white birds in F2 population. But the reverse is true for the grey feather colour and black feather colour for both of these colours, Silkie allele is increasing and its higher proportion giving the feather grey and black rather than off white colour. Same is the case with the light brown colour with increased proportion of Silkie allele but its locus is mapped on chromosome 4.

6.5.9 Feather Pattern

In the present study, a significant locus for feather pattern was also found after removing animals carrying the dominant white allele, barring, speckled and lacing. QTL for the all feather patterns were found on E22C19W28. Significant QTL ($P<0.01$) for barring of both neck and wing region were found at 25cM with the F-ratio being 26.19 and 25.31 respectively and the 95% C.I. was between 13 and 28cM and 16-28cM respectively.

The trait of lacing of neck, wing and back feathers had a significant locus ($P<0.01$) on linkage group E22C19W28 at 27, 27 and 30cM with F-values of 22.18, 19.16 and 19.75 respectively. The 95% C.I. for the feather patterns in the neck and wing region ranged from 0-29cM and in the back region from 0-27cM.

The last feather pattern studied was speckled and its locus was also found on the linkage group E22C19W28 where the dominant white loci resides. The significant locus ($p<0.01$) for speckled neck and speckled back was found at 28cM but speckled wing QTL was at 29cM.

The additive and dominant effects of feather patterns are in Table 6.2. The additive and dominant effects of barring and lacing in the region of neck, wing and back region is negative indicating increased proportion of Silkie alleles. In case of speckled the loci are showing significant dominant effects.

Flanking marker for all feather patterns, barred, speckled and lacing was rs16705784 and ROS0054 indicating that genes controlling these trait are present in between these two makers.

6.6 Discussion

6.6.1 Comb type

Initially, with low density markers, QTL for comb type was found on chromosome 7 with F value 291.64.The peak position of comb type was at 0cM coincident with the ADL0279 marker. 15 SNPs were genotyped including 3 targeted SNPs obtained by blasting the next generation sequences of White Leghorn and Silkie, which increase the F- value upto 798. Addition of SNPs gives 95% confidence interval (C.I.) of the genetic locus in between 74-80 cM.

After the addition of SNPs markers the length of the confidence interval for comb type was very small at 6 cM which meant that the number of genes potentially controlling the trait is in this region was very small. The potential genes in this region are IHH, MNR2, MIR375, CRYBA2, MIR1788, WNT10A, WNT6, PPKAG3, RNF25 and BCSIL. Examination of these potential genes in this chromosome region suggested some promising genes which are believed to be involved in developmental biology are WNT6, WNT10A (Schubert et al., 2002, Schmidt et al., 2007). After genotyping three SNPs it was found that peak position of genetic locus resides in between SNPs of WNT6 and WNT10A. Further work, described in chapter 7 has been done to examine the function of these genes with the development of rose comb.

Dorshorst et al.(2010) studied different types of comb but initially they did not find significant regions for Rose (R) or Pea (P) combs in either cross when comb type was considered a single trait with up to 6 categories (Single, Rose, Pea, Walnut,

Duplex, and Mixed). To overcome the problem of misclassification of trait they classified Walnut comb phenotype birds as putative carriers of at least one dominant allele or the recessive allele at R and P. This resulted in a region of chromosome 7 detected as having significant association with Rose comb in the region of 16.9–22.4 Mb. They did not find obvious candidate genes in this region.

As our QTL of comb type on chromosome 7 has also significant dominant effect and this is in agreement with the finding of earlier studies of Bateson and Saunders (1902) who first reported the inheritance of this trait and found that rose comb is an autosomal dominant trait. Contrasting results were observed by Wright et al. (2009) who found that a copy number variation in intron 1 of SOX5 on chromosome 1 is responsible for controlling pea type comb in chicken. The reason for these contrasting results is due to difference in the type of comb, in the present study the type of comb carried by Silkie is rose comb.

Cochez(1951) and Ponsignon (1951b) found that reproductive performance of rose comb birds was poorer than that of single comb birds. Crawford and Smyth (1964) conducted extensive studies of the reproductive efficiency of birds of three comb genotype (RR, Rr, rr) and found that low fertility is obtained from homozygous rose comb male(RR). In the present study, we did not work on the association of rose comb with fertility problems.

6.6.2 Crest type

A highly significant genetic locus ($P<0.01$) for Crest type was found on linkage group E22C19W28 at 7cM with an F-value of 120.9. The confidence interval

spanned a region of around 13cM. The additive and dominant effect of crest type is -0.44 and 0.39 respectively.

As explained earlier in chapter 5, the trait has a significant additive effect and negative value indicate that increasing allele is coming from Silkie. Initially, one microsatellite marker, ROS0054, was mapped on this chromosome giving peak position of QTL at 0cM. The addition of three informative SNP markers and one microsatellite markers allowed the definition of the peak position on 7cM.

The confidence interval of the QTL ranged from 4-17cM (13cM). Most of the genes in this confidence interval are related to keratin including KRT8 (Keratin 8), KRT4 (Keratin 4), Q6PVZ3_Chicke (type II alpha-keratin IIC) LOC426897 (similar to keratin), K2CO_Chick (Keratin type II), KRT80 (keratin 80). From this list of genes present in the confidence interval of crest type QTL, it seems that keratin is likely to be a potential candidate for the formation of crest. The Crest is a feather feature. It was known that alpha keratins have an important role in establishing the structure of feathers (Ng et al., 2012). It was also found in the literature that crest is associated with an abnormality of skull known as cerebral hernia(Brothwell, 1979). Keratin 4 is also known as cytoskeletal 4 and is a basic and neutral protein and is expressed during differentiation of epithelial tissue. Mutation in this gene is associated with the abnormalities of skin condition in human (Zhang et al., 2009). Although in the present study we did not record cerebral hernia but it may be related to cerebral hernia in a population of White Leghorn and Silkie. There might be a possibility that ectopic expression of current gene is also responsible for some type of abnormalities in chicken.

Results of the current study contradict with Wang et al. (2012). They correlated crest phenotype with ectopic expression of HOXC8 in the cranial skin and indicate the presence of the gene on linkage group E22C19W28. In the new assembly of chicken genome on UCSC site, (Nov 2011, ICGSC Gallus_gallus 4.0/galGal4), HOXC8 gene is still present on unknown chromosome and not mapped to any linkage group. Their result also contradicts with previous reports based on fluorescent in-situ Hybridization (FISH) that indicated that the HOXC cluster is located on chicken chromosome 1 and also the NCBI GenBank description about the position of chicken HOXC8 (Gene ID: 395711, at NW_003778389.1, from 237 bp to 1871 bp) (Ladjali-Mohammedi et al., 2001).

Results of present study indicate that the locus for Rose comb is present on chromosome 7 while the crest type locus is on chromosome E22C19W28 indicating that different genes are involved in controlling the traits of comb type and crest type. These results are in contrast to the finding of Jull (1930) who studied the association of rose comb with crest type in a cross of White Leghorn and Silkie and concluded that the same gene is controlling these two traits. This trait is also considered to be associated with cerebral hernia (Bartels, 2003, Ga et al., 2010, Frahm et al., 2001).

6.6.3 Fibromelanosis

The genome wide significant genetic locus ($P \leq 0.01$) on chromosome 20 appeared with F value 7.56, 10.4 and 9.81 for the integument, meninges and for the muscle membranes. The peak position of this QTL was found in between markers rs15176962- rs15177166. The significant additive effect of -0.23, -0.62 and -0.40 were found for the integument, meninges and muscle were found respectively which

indicate wide variation of trait between both of these breed. Negative value indicates that increasing allele is coming from Silkie.

Results of the present study are partly in agreement with Dorshorst et al. (2010) who did a detailed study on the fibromelanosis trait. They found association of fibromelanosis with a locus on chromosome 20 but they also found significant association of sex linked inhibition of dermal melanin (*Id*) on Z chromosome and but in our study we did not find any locus for fibromelanosis on the Z chromosome. In a follow up study of fibromelanosis, Dorshorst et al. (2011) found that the presence of the dominant FM allele results in extensive pigmentation of the dermal layer of skin and the majority of internal connective tissue. They also identified that causal mutation of FM is due to inverted duplication and junction of two genomic regions separated by more than 400 kb in wild-type individuals. One of these duplicated regions contains endothelin 3 (EDN3), a gene with a known role in promoting melanoblast proliferation.

Results of the current study are also in contrast with the earlier studies. Bateson and Punnet (1911) quoted by Hutt (1949) that black colour in internal organ is inhibited by a sex linked gene carried in Brown Leghorns. Dunn and Jull (1927) found a similar inhibitor in White Leghorns and concluded that it was either identical with the gene *Id*, inhibiting dermal melanin. No evidence of sex linked inheritance of trait was found in the current study although we have short coming that number of birds studied for the trait are quite small and F_2 population are only females.

6.6.4 Feathered leg

Initially when this trait was analysed with low density makers, there was appearance of three putative QTL on chromosome 5, 9 and 13. Although for fine mapping of genetic locus on these chromosomes, 30 SNPs markers were added on chromosome 5 and 28 SNPs markers on chromosome 9 and 28 SNPs markers were added on chromosome 13. After the addition of SNPs, the QTLs on chromosome 5 and 9 disappeared but there is still existence of QTL on chromosome 13 and its confidence interval (CI) is reduced to 20cM. Initial finding were in agreement with Somes (1992) in which he correlate this trait with several loci and also found the quantitative nature of this trait rather than Mendelian trait. But the Silkie feathered leg is thought to be caused by one or two incompletely dominant loci. The current locus on chromosome 13 for feathered leg has significant dominant effect. So presence of genetic locus of feathered leg on chromosome 13 is partly in agreement with the conclusion of Somes (1992) about the inheritance of Silkie feathered leg.

After fine mapping, the presence of one genetic locus on chromosome 13 is in agreement with the finding of Dorshorst et al (2010). They studied feathered leg and vulture hock together and found the association of both of these two traits with rs14999343 on chromosome 13. But they are unclear which trait is truly associated with this region. This SNP is present in the confidence interval of current QTL on chromosome 13 but it does not lie in the peak position of QTL.

6.6.5 Feather colour

The genetic loci for the white feather colour in the region of neck wing and back appeared on Z chromosome while highly significant loci for off white, grey and black feather colour in the region of neck, wing and back were found on chromosome E22C19W28. A locus affecting brown colour was found on chromosome 4.

In the present study, presence of white feather colour loci on the Z chromosome is in accordance with the findings of Gunnarsson et al. (2007). They found a mutation in the SLC45A2 gene and concluded that mutation at this locus cause an almost complete absence of eumelanin. This gene is present in the confidence interval of the current locus. Kerje et al. (2004) found that Dominant white is associated with 9bp insertion in the exon 10 of PMEL17 Presence of off white and grey colour locus were also found on linkage group E22C19W28 also indicate that other than dominant white locus at the location of PMEL17 (Kerje et al., 2004), there is still some other genetic difference controlling the feather colour in chicken.

6.6.6 Feather Pattern

In the present study, we also found significant QTLs for feather pattern on linkage group E22C19W2. Again it was hypothesised that the presence of a feather pattern locus on linkage group E22W19W28 was due to the presence of the dominant white locus (Kerje et al., 2004). Again after reanalyses for all of the feather pattern after removal of animals carrying the dominant white allele we found that barring, speckled and lacing that the loci for all feather patterns remained on E22C19W28

(P<0.01) at a near identical position. Flanking marker for all feather pattern, barred, speckled and lacing was rs16705784 and ROS0054 indicating that genes controlling these trait are present in between these two makers.

6.7 Conclusion

From the current results we found small confidence interval of comb type on chromosome 7. There are certain promising genes like WNT6, WNT10A. The peak position of comb type is in between markers of WNT6 and WNT10A. Both of these genes are important as their role is in developmental biology is well documented. These genes are further investigated with reference to comb type in next chapter. QTL found for other trait crest type, feather type, feathered leg, feather colour and pattern and fibromelanosis and skin colour are also important. QTL for feather type found on chromosome 3 with confidence interval of 3cM also give a better opportunity of limited genes and further dissection of these genes give the exact mutation and also helpful to know the biology of Silkie feathering in the Silkie chicken. Due to limited resources and time, all traits are not investigated in depth.

Chapter 7 Functional genomic exploration of WNT gene as candidate for comb type

7.1 Introduction

Comb plays a major role in the chicken, it helps to regulate the temperature of the body and also absorbs light and it maintains the social structure of the flock (Folsch et al., 1994). The comb is a vascular, red cutaneous structure attached in a sagittal plane to the dorsum of the skull of domestic fowl. It consists of a base attached to the skull, a central mass called the body, a backward projecting blade and upward projecting points. As chickens do not sweat it is believed that they can dissipate 15% of their body heat through comb and wattles (Sturkie, 1965).

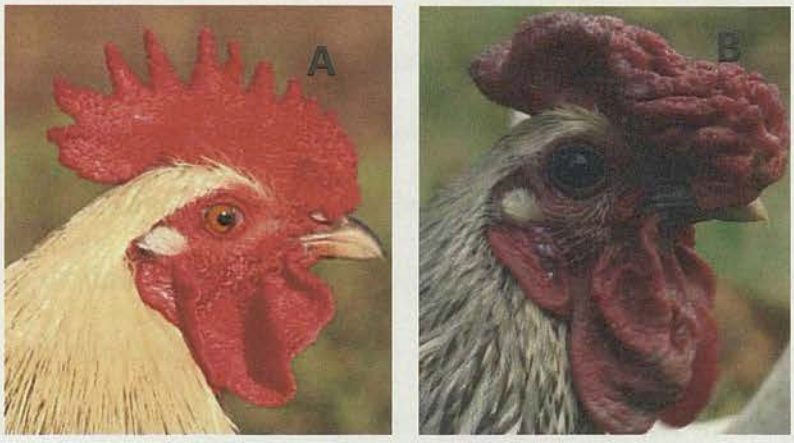


Figure 7-1 showing Single comb White Leghorn (A), Rose comb Silkie (B)

Chicken comb is primarily composed of collagen and hyaluronan, which are produced by chondrocytes. These cells are formed through the condensation and differentiation of mesenchyme cells in the chondrogenesis pathway (Wright et al., 2009). WNT (Wingless type) signalling pathways have well established role in the chondrocyte differentiation, proliferation and maturation during embryonic development (Yates et al., 2005). In the chicken embryo, most of the skull and the entire facial and visceral skeleton arise from Neural crest cells (Creuzet et al., 2005) .

These cells are source of patterning information. Neural crest cells are a transient, multipotent, migratory cells that develop during embryogenesis and are unique to vertebrates (Huang and Saint-Jeannet, 2004). After induction, neural crest undergoes epithelial-mesenchymal transition and delaminates from the neural folds (Schmidt et al., 2007). Their subsequent migration into the periphery allows these cells to gives rise to a diverse cell lineage including melanocytes, craniofacial cartilage and bone, smooth muscle, peripheral and enteric neurons (Bronner-Fraser, 1994). Due to this reason neural crest cells are sometime referred to as the fourth germ layer(Schmidt et al., 2007). A map of neural crest cells migration towards face in avian embryo during embryonic development is shown in Figure 7-2.

Neural crest induction and migration involved multiple signalling pathways and transcription factors, and occur in two phases from gastrulation to neurulation. In the first phase, FGF and WNT signalling induce Neural Crest progenitors at the border of the neural plate. In the second phase, BMP, WNT, and Notch signalling maintain these progenitors through the expression of neural crest markers(Stuhlmiller and García-Castro, 2012). The signalling of these pathways between epithelia and mesenchyme regulate the development of tissues and organs in the craniofacial development (Abzhanov et al., 2007).

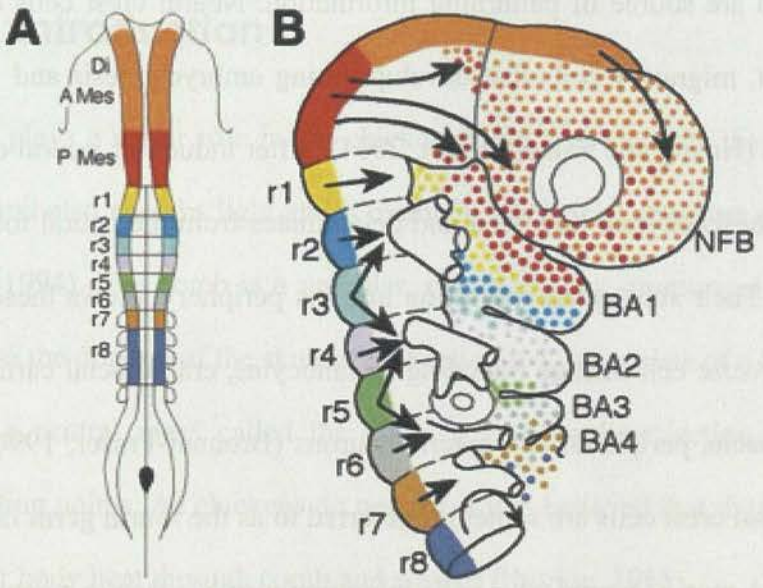


Figure 7-2 (A) Presumptive diencephalic, mesencephalic and rhombomers (r1-r8) of neural fold in the avian embryo (B) Migration map of neural crest cells towards face during avian embryonic development (Creuzet et al., 2005).

WNT signalling (including WNT6, WNT10A) have a major role in the development of neural crest cells and allowing the expression of neural crest specifier which controls neural crest cells migration and differentiation (Schmidt et al., 2008) . It is believed that WNT6 has a profound role as a typical ectodermal neural crest inducer in quail-chick chimeras and also helps cranial neural crest to migrate their final location towards head (Schubert et al., 2002). WNT10A expression pattern in the chick during development was also studied (Narita et al, 2005). WNT10A is G-protein coupled receptor binding protein and reported to has a significant role in the skin development, epidermal morphogenesis, neural crest differentiation, anterior and posterior pattern specification in human (Yates et al., 2005). On the basis of a significant role of WNT6 and WNT10A in the neural crest induction and migration,

current study was designed to prove the results of chapter 6 where it was found that the peak of the genetic locus explaining comb type was found in the markers of WNT6 and WNT10A genes.

7.2 Objective

To test the hypothesis that the WNT genes (WNT6, WNT10A) are candidates for the genetic variation in comb type

7.3 Material and Method

7.3.1 Preparation of Embryos for Whole mount *In-Situ* Hybridization

Whole mount *In-situ* hybridization was performed in different embryonic stages, 20 HH, 21 HH, 23 HH, and 25HH separately for White Leghorn and Silkie. Due to fertility problems with the Silkie it was not easy to get pure embryos from the Silkie birds so eggs of F1 (Silkie x White Leghorn) were used in place of Silkie embryos in some cases. In each stage, at least 10-15 embryos of respective stages were used. Detail of preparation of embryos for *In-situ* hybridization is in chapter 2, section 2.14.2.

7.3.2 Probe synthesis of WNT6 and WNT10A for Whole mount *In-Situ* Hybridization

Detail of probe synthesis of WNT6 and WNT10A is in chapter 2, section 2.13.

7.3.3 Whole mount *In-Situ* Hybridization

Detail of whole mount le mount *In-Situ* Hybridization is in Chapter 2, section 2.14.

7.3.4 Western Blotting of WNT6

Detail of western blotting of WNT6 is in chapter2, section 7.3.4.

7.4 Result

Current study is based on the finding of chapter 6 the peak of the genetic locus explaining comb type was in between markers in the WNT6 and WNT10A genes. Single comb is the characteristics of White Leghorn and rose comb is the characteristic of Silkie. To check the expression of WNT6 and WNT10A on the development of comb and also to see the difference of expression of these genes in the White Leghorn and Silkie embryos, whole mount *in-situ* Hybridization was performed in various embryonic stages of both of these breeds.

7.4.1 Expression of WNT6 and WNT10A in White Leghorn and Silkie/White Leghorn embryos

To see the expression of WNT6 in White Leghorn and Silkie embryos in the head region from where comb arises, Whole mount *in-situ* hybridization was performed in different embryonic stages, 20- 21 HH, 23 HH and 25 HH separately for each breed (Hamburger and Hamilton, 1992).

Ectodermal expression of WNT6 was found on the roof of the mesencephalon, pretectum and dorsal thalamus (Figure 7-3). This expression was found in both White Leghorn and Silkie embryos but in case of White Leghorn, expression was greater as compared to Silkie (Figure 7-4).

Expression pattern of WNT10A was also studied by *in-situ* hybridization in different embryonic stages of 20-21 HH, 23 HH and 24 HH in White Leghorn and sillies embryos.

No expression of WNT10A was observed in the head and in the face portion of any of the embryonic stages.

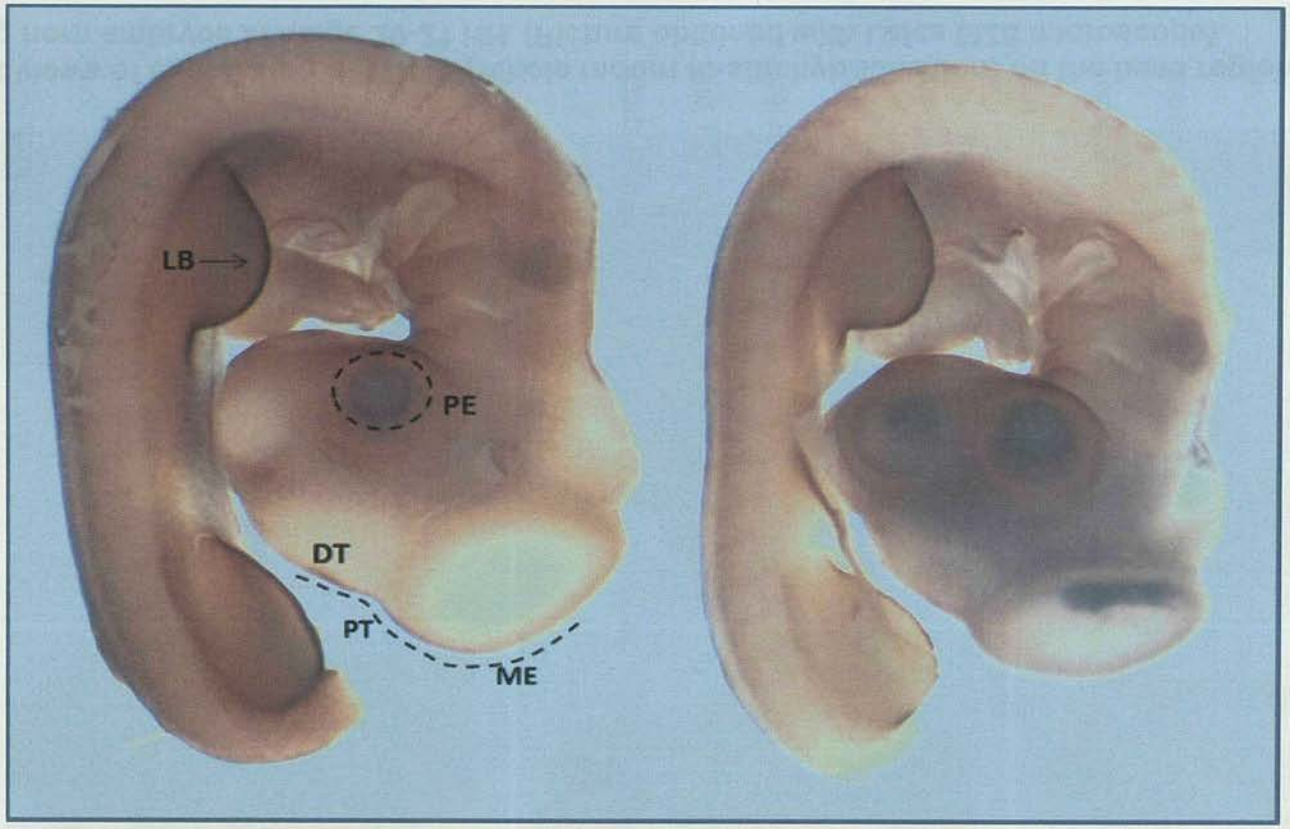


Figure 7-3 Whole mount *In-situ* Hybridization of WNT6, White Leghorn embryo (right side), Silkie embryo (left side). Expression of WNT6 (Dark line) is present in Roof of Mesencephalon (ME), protectum (PT) and dorsal thalamus (DT). Expression was also observed around periphery of eyes (PE) and limb buds (LB). (Picture obtained with Leica MZ8 microscope)

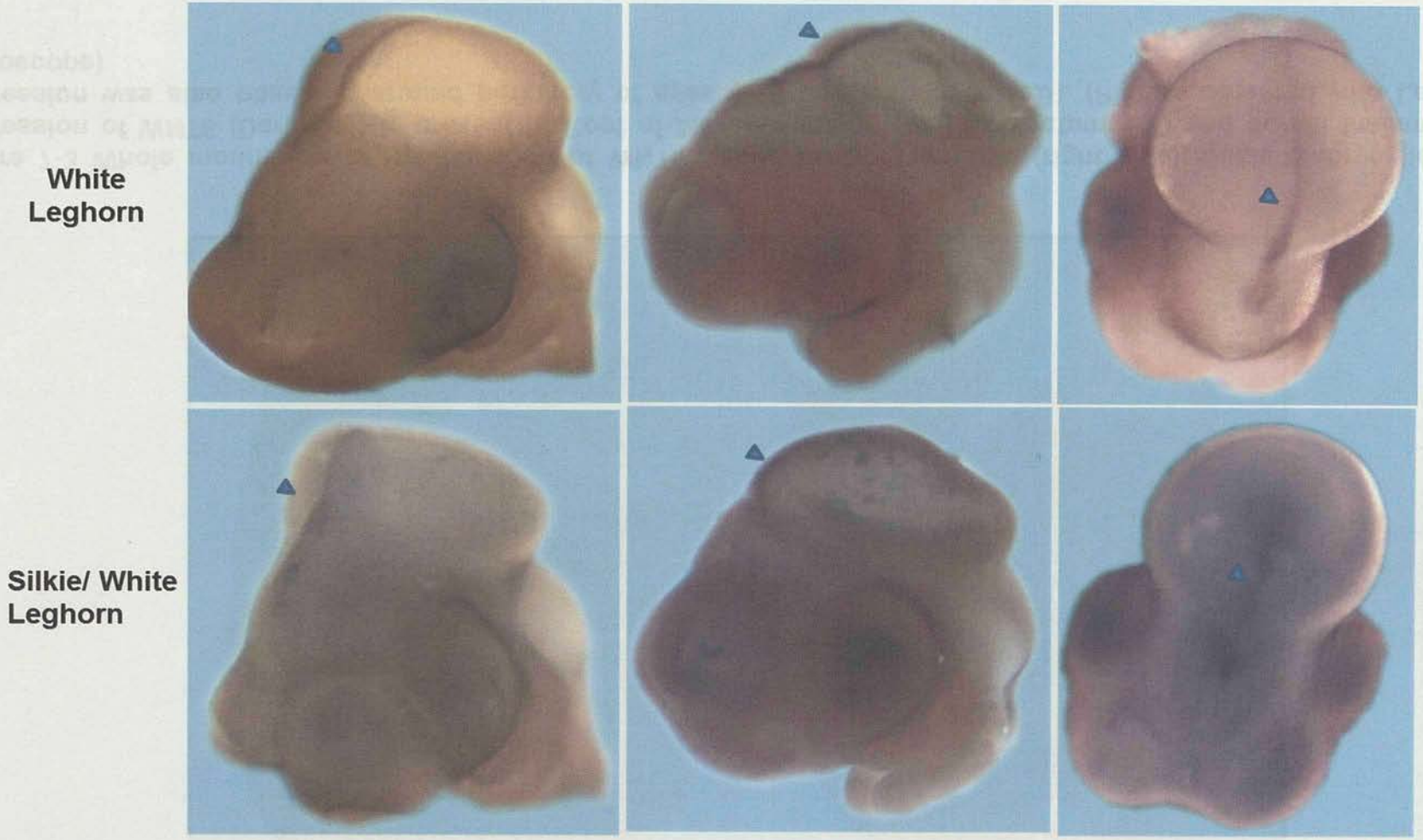


Figure 7-4 Different views of expression of WNT6 (Whole mount *in-situ* hybridization) on the head region of White Leghorn and Silkie/White leg horn embryos at stage 20-21 HH. (Picture obtained with Leica MZ8 microscope).

7.4.2 Protein levels of WNT6 in White Leghorn and Silkie

By *In-situ* hybridization, it was found that there was expression of WNT6 in the head region of both White leg horn and Silkie/White Leghorn embryos during the stage of 20-21 HH. To further prove the hypothesis that WNT6 could be driving the patterning of the comb and that difference in the amount of WNT6 protein could account for the difference seen in their comb structure between breeds, a western blot was done.

Western blot against WNT6 with White Leghorn and pure Silkie embryos was performed at stage 20-21 HH. A difference in the intensity of signal for WNT6 protein was observed between White Leghorn and Silkie and is shown in Figure 7-5 and Figure 7-6. Tubulin is a control gene. One pool of White Leghorn and Silkie heads at stage 20-21 HH was used in triplicate (Figure 7-5, Figure 7-6).

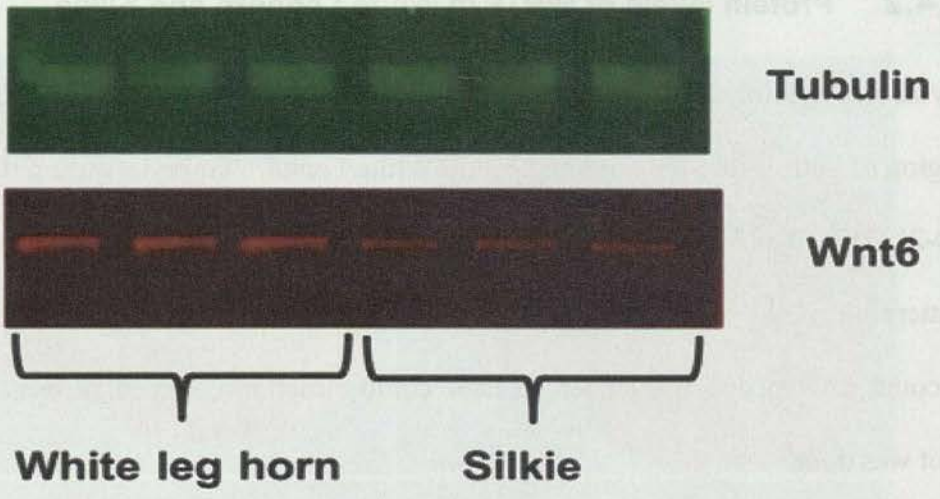


Figure 7-5 Western blot against WNT6 protein at embryonic stage of 20–21 HH of White Leghorn and Silkie Embryos. Tubulin was used as control.

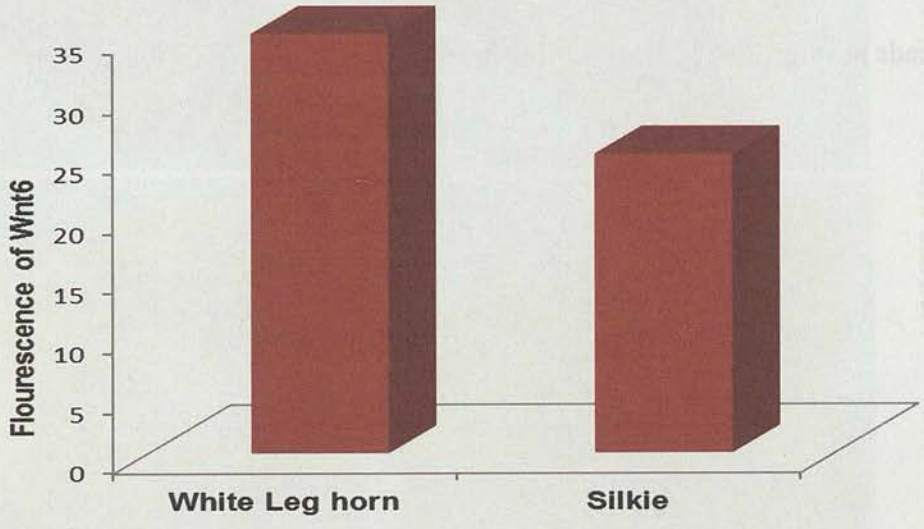


Figure 7-6 Difference of fluorescence of WNT6 band in White Leghorn and Silkie head during the western blot

7.5 Discussion

A series of *in-situ* was performed to investigate the difference of WNT6 and WNT10A in both breeds of White Leghorn and Silkie at different embryonic stages. By whole mount *in-situ* Hybridization, it was found that WNT6 is showing expression on the top of the head corresponding to the roof of mesencephalon, pretectum and dorsal thalamas (Figure 7-4) of embryos at stage 20-21 HH. WNT10A did not show any expression in this region. From *in-situ* experiment, it was found that WNT6 is the gene which is expressed in the cells related to the comb as its showing correct spatiotemporal expression in the chick embryos. This is in line with the findings of Me´sa´r et al (2008) and Lawrence (1968) who grafted comb primordial from different ages at various locations and found that cells give rise to comb are determined before the embryonic stage of 24 HH.

A Western blot demonstrated a difference of protein level of WNT6 between breeds in this region. This suggests that WNT6 is the gene that might be involved in the patterning of the comb and could cause the difference in the variation of structure in both of these breeds. Further replication is however required. This conclusion is however supported by the well-established role of WNT6 in the induction and migration of neural crest cells and their further involvement in the formation of craniofacial cartilage and bone, smooth muscle (Schmidt et al., 2008, Geetha-Loganathan et al., 2009, Garcia-Castro et al., 2002).

WNT signalling pathways has well established role in the chondrocyte differentiation, proliferation and maturation during embryonic development (Yates et

al., 2005) and it is known that layers and connective tissues of combs are composed of collagen and hyaluronan which are formed from mesenchyme cells through chondrogenesis pathway (Wright et al., 2009). Lawrence (1968) did grafting of single-comb primordia to the neck region without beak mesenchyme, and found that it lost the serrated single ridge morphology and proved that morphology of the comb was under control of the mesenchyme.

From the results it was also found that changes in the expression and protein level of WNT6 will not only affect the determination and initial stages of the comb development but rather the development of comb shape and is likely to change the identity of the mesenchyme underlying both the comb. Schmidt et al. (2007) showed that neural crest induction requires signalling from WNT6 and its expression is connected to tissue re-architecturing such as epithelial to mesenchymal transformation. Experiment on comb primordium development showed that the comb shape is directly dependent on instructive signals derived from the underlying mesenchyme (Lawrence, 1971, Lawrence, 1968).

7.6 Conclusion

1. From the expression of WNT6 on the roof of mesencephalon, pretectum and dorsal thalamus at the embryonic stage of 20-21 HH and potential difference of WNT6 protein level in both of breeds (White Leghorn and Silkie), it is concluded that WNT6 may be the potential candidate responsible for the genetic variation of comb

2. It is also concluded that WNT10A was unlikely to be involved in the comb development because of lack of its expression.

Chapter 8 General Discussion and Conclusion

8.1 Introduction

The current study was conducted principally to find the genetics of incubation behaviour but the breeds used to study this trait also provide a good opportunity to study genetics of several Morphological traits including comb type, crest type, feather type, feathered leg, comb colour, fibromelanosis, skin colour, leg colour and feather colour and pattern etc.

Incubation behaviour is a major problem in conventional breeds of chicken. In developing countries, despite the impact of rural poultry in poverty alleviation and significant contribution in national economy, there has been a lack of research to improve the efficiency of traditional poultry production (Kitalyi, 1997). Traditional village poultry production is not efficient and has low productivity because of incubation behaviour (Anjum et al., 2012). On the other hand, these breeds have great potential for disease resistance (Anjum et al., 2012, Bhatti and Sahota, 1996) and potentially interesting DNA to be incorporated into commercial founder lines. However, because of the success of commercial production in eliminating incubation behaviour by genetics and the fact that keeping hens in cages has reduced the problem in intensive production. There has been lack of research into the reasons of low production (e.g. incubation behaviour) of traditional poultry and these traits have not been subject to successful genetic improvement. In summary, traditional poultry production is making a difference to poverty alleviation, but there is scope for even greater impacts of poultry to the rural economy if the early onset of incubation behaviour could be ameliorated (Scanes, 2007). Some aspect of incubation behaviour

has been studied in the past. But this the first time, a comprehensive study was conducted to study the genetic basis of incubation behaviour.

Morphological traits have also great economic importance and improvement in these traits can also have economic implications. Commercial breeding companies may find markers for comb type might help them to reduce damage of combs from fighting and pecking in cockerels, it is seen as a welfare issue (Millman, 2002). The importance of crest in chicken cannot be ignored as this trait in homozygous form effects the fertility of male chickens (Wang et al., 2012). In certain parts of the world, consumers are willing to pay higher prices for tasty meat and for the appearance of the birds (Fanatico and Born, 2002, Zhou, 2002). According to consumer demand, producers need to look at the bird appearance, and adapt breeding strategies to match the appearance to the market of the country. These traits are almost like trademarks and may improve the acceptance of improved breeds if they are kept. In southern China, yellow skin and shank has been considered as indicator for high quality of meat and good luck (Yang and Jiang, 2005). In the live market, consumers pay much more attention to colour of plumage, skin and shank as well as redness and size of comb. Black skin and shank are widely accepted as a nutritive and tonic food for the consumer (Yang & Jiang 2005).

8.2 Reproductive trait/Incubation behaviour

Two significant QTLs have been detected for the trait of incubation or maternal behaviour. The first QTL for incubation status on chromosome 5 at 100cM was significant at 5% genome level and it covered a period of 52 weeks. Other QTL forearly incubation behaviour (25-30 weeks) on chromosome 8 at 21cM was

significant at the genome wide 1% level. Targeted SNPs were used to refine the estimated position of the loci which in the case of the QTL on chromosome 5 was reduced to a 95% CI of 45 cM between flanking markers rs13587819 and MCW0032 and in case of early incubation behaviour 95% CI reduced from 89.5 to 45 cM. The remaining QTL loci for incubation status on chromosome 1, 18, 19 and E22C19W28 were suggestive. For early incubation behaviour, suggestive QTLs were found on chromosome 1 and 26. (Chapter 3 and Chapter 4).

8.2.1 Incubation status and Selective Sweep

The peak position of the QTL on chromosome 5 (Chapter 4) coincides with the location of the largest selective sweep observed in a study comparing domesticated breeds versus red jungle fowl (Rubin et al., 2010). In this paper a mutation in the thyroid stimulating hormone receptor (TSHR) gene was highlighted as being a possible target of the selective sweep, however evidence for its mode of action is awaited. However the Silkie and White Leghorn do not differ at this SNP (Leif Anderson personal communication) but it remains that the region must have importance for the domestication of poultry and further selection on the loci may have profound effects on the biology of chickens. The QTL for incubation status on chromosome 5 has number of genes involved in the early events of the transduction of stimulatory photoperiodic information to the reproductive system (Nakao et al., 2008a) (Figure 8-2). These genes are TSHR which is the receptor for thyroid stimulating hormone, DIO2 (deiodinase, iodothyronine, type II) and DIO3 (deiodinase, iodothyronine, type III) (Ono et al., 2008). The level of TSHR is elevated within the first long photoperiod in the pars tuberalis and two deiodinases,

DIO2 and DIO3, which are respectively stimulated and inhibited by the activation of the TSHR (Nakao et al., 2008b, Yoshimura, 2010). This results in an increase in the conversion of T4 to the biologically active thyroid hormone T3 in the brain (Figure 8-1). Thyroid stimulating hormone with its receptor (TSHR) in the thyroid gland plays a crucial role in the pituitary-thyroid axis of all the vertebrates and controls numerous metabolic process in the body(Grommen et al., 2006). Although in the present study, data on the feed consumption was not recorded but it is noted that birds eat hardly during incubation behaviour and there is dramatic changes in the metabolism due to changes in the TSHR. It is striking but perhaps coincidental that the remaining thyroid hormone deiodinase (DIO1) is to be found in 95% CI of the second significant QTL on chromosome 8. Clearly these genes are part of the thyroid hormone system controlling metabolic activity.

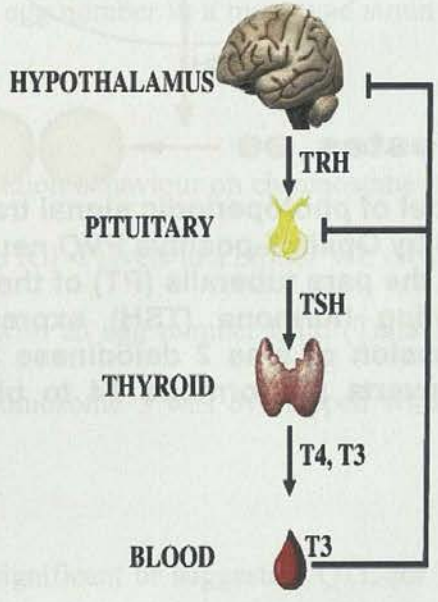


Figure 8-1 Normal Thyroid hormonal system controlling metabolic activity in the body (Sirakov and Plateroti, 2011).

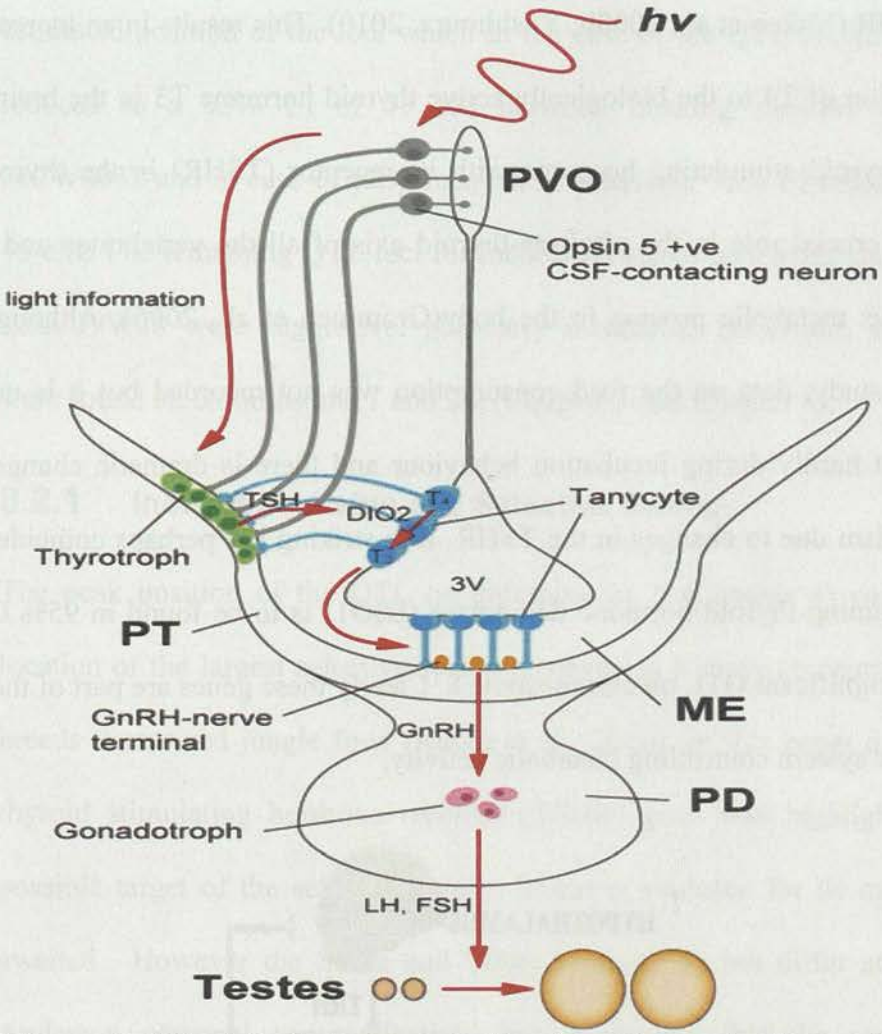


Figure 8-2 Model of photoperiodic signal transduction pathway in birds. Light detected by Opsin 5-positive PVO neurons that contact the CSF is transmitted to the pars tuberalis (PT) of the pituitary gland and induces thyroidstimulating hormone (TSH) expression in the PT. PT TSH induces expression of type 2 deiodinase (DIO2) in the third ventricle (3V). DIO2 converts prohormone T4 to bioactive T3 (Nakanea et al., 2010).

8.2.2 Coincidence with other thyroid hormone system QTL and reproductive QTL

The link to the thyroid hormone system is supported by the study of the coincidence of position of the QTL in this study from the data base of poultry QTLs (Hu and Reecy, 2007). In particular the QTLs on chromosome 1 for incubation status and for early incubation behaviour are in the region which is overlapped with QTL for levels of T3 and T4 and their ratio, which is dependent on the deiodinase enzymes, and IGF-I levels in a broiler-Fayoumi cross (Zhou et al., 2007). A QTL at this position for IGF-I levels was also identified in a study using high and low growth lines (Nadaf et al., 2009). The loci is also overlapped with age at first egg in a cross between a Rhode Island Red and White Leghorn line (Tuiskula-Haavisto et al., 2004) and in a study using the same WL strain in the current study and a broiler line (Podisi et al., 2011) and egg number in a meat type strain WL cross (Hansen et al., 2005).

The QTL for early incubation behaviour on chromosome 8 is overlapped with QTL for T3, T4, their ratio and IGF-I levels in a broiler WL cross (Zhou et al., 2007). This loci is also overlapped with an egg number QTL (Tuiskula-Haavisto et al., 2002). Finally the QTL on chromosome 5 was overlapped with an egg production QTL (Atzmon 2007

Therefore, of the eight significant or suggestive QTL for incubation status or early incubation behaviour four of them, on chromosomes 1, 5 and 8, have a links with the thyroid hormone system. In the T3/T4 and IGF-I QTL studies on chromosome 19 and linkage group E22C19W28 were not covered. This clearly defines that the

thyroid hormone system may be important but it does not illuminate why it should be important for incubation behaviour but rather suggests why effort should be focussed on this pathway.

8.2.3 Domestication and Incubation Behaviour

Under domestication and selection pressure Incubation behaviour has been eliminated in certain breeds of chicken, notably the White Leghorn but it still present in many breeds of poultry including Red Jungle fowl (Collias and Collias, 1967).. This revolutionary technological development of Egyptians artificial incubation methods was a pre-requisite for the development of lines of chickens that do not display incubation behaviour and the breaking of the link between maternal behaviour and reproduction (Megyesi and Henson, 2011). As incubation behaviour is associated with a cessation of reproduction for many weeks it is clearly desirable to remove this trait to produce highly productive egg laying strains. Due to loss of incubation behaviour in modern strain the number of egg per year is increased up to 300 eggs per year (Moreng and Avens, 1985, Romanov and Weigend, 2001)

In contest to current study on incubation behaviour it is found that the mode of action of the QTLs on chromosome 5, 1, 18, 19 and E22C19W28, indicate the White Leghorn allele is promoting incubation behaviour or in other words it can be said that heterozygotes performance is better than homozygotes. We believe some of these are due to changes in fertility which increases the chance of incubation behaviour being observed. Therefore the QTL on chromosome 8 for early incubation behaviour is most likely to be a locus that has an effect directly on incubation since it is both for early incubation, where duration of reproductive activity is not important

and the inheritance on chromosome 8 is as expected with the Silkie allele promoting incubation behaviour. Similarly the QTL on chromosome 1 has dominance mode of inheritance where dominant White Leghorn allele making the birds less broody and may be directly for incubation behaviour. The locus on chromosome 5 may have importance both for production and fertility but also for the maintenance of incubation behaviour, possibly utilising common components in the basal hypothalamic TSH to T3 production pathway which includes TSHR and the deiodinase enzymes (Nakao et al., 2008a). It is presumed that selection for long time decreases incubation behaviour in White Leghorn but it seems that this procedure is incompatible with the loss of incubation behaviour. Because of the coincidence of the loci on chromosome 5 with the site of the strongest selective sweep in poultry, the TSHR, and the coincidence of the QTL on chromosome 1 and 8 with thyroid hormone activity QTL it would appear that the thyrotrophic axis may be critical to the loss of incubation behaviour during domestication.

8.3 Morphological traits

A genome wide Significant (P<0.1) genetic locus for the comb type was found on chromosome 7 with F-value 798. Fine mapping of comb type QTL with SNPs markers gives a confidence Interval (C.I) of 6cM (Chapter 6). Initially with microsatellite markers which gives small number of genes controlling the traits (Chapter 5). By fine mapping it was found that the peak of the comb type QTL is in between markers of WNT6 and WNT10A genes. The importance of these genes in developmental biology is well known(Geetha-Loganathan et al., 2009) . The WNT gene family consists of structurally related genes which encode secreted signalling proteins. These proteins have been involved in several developmental processes, including regulation of cell fate and patterning during embryogenesis (van Amerongen and Nusse, 2009).

To test the hypothesis that WNT gene is candidate for genetic variations of comb type, whole mount *in-situ* Hybridization (at different embryonic stages) and western blot of WNT6 was done at stage 20-21HH (Chapter 7) . WNT6 appears to have a difference of expression on the roof of mesencephalon, pretectum and dorsal thalamas at the embryonic stage of 20-21HH and a potential difference of WNT6 protein level was found in between breeds (White Leghorn and Silkie). From these results, it is concluded that WNT6 is a potential candidate responsible for the genetic variation of comb type (Chapter 7) and can be investigated further. WNT signalling have a major role in the development of neural crest cells and allowing the expression of neural crest specifier which controls neural crest cells migration and differentiation (Schmidt et al., 2008) . It is believed that neural crest cells are the

cells which give rise skull, facial and visceral skeleton of the chicken including the chicken comb , It is found that WNT6 has a profound role as a typical ectodermal neural crest inducer and also helps cranial neural crest to migrate their final location (Schubert et al., 2002). Schmidt et al. (2007) showed that neural crest induction requires signalling from WNT6 and its expression is connected to tissue re-architecturing such as epithelial to mesenchymal transformation. Experiment on comb primordium development showed that the comb cells are derived from mesenchyme (Lawrence, 1971, Lawrence, 1968).

A recently published paper has confirmed the presence of the genetic loci for rose comb to chromosome 7. They suggested that the comb phenotype is caused by a large inversion of ~ 7.3Mb (Imsland et al., 2012). This causes the relocalisation of the MNR2 homeodomain protein gene thus presumably separating it from its natural enhancers and promoter which leads to transient ectopic expression of MNR2 during comb development. WNT6 is outside the boundary of this inversion and is present on the opposite strand. It is however possible that a enhancer or other control elements has been moved by the inversion if present in the Silkie. In the present study, a series of strategies were adopted to find the gene causing the genetic variation in comb type including, mapping of QTL, Fine mapping of QTL and further sequencing of SNPs in the peak position. After finding the peak position in between markers of WNT6 and WNT10A, the expression pattern of these two genes was studied in White Leghorn and Silkie chicken embryos by whole mount *in-situ* Hybridization and western blot. At the end it is concluded that WNT6 is a potential candidate for the genetic variation of comb type. Although, results of the current study are different than the above study but it's equally validated and there is

possibility that both MnR2 and WNT6 are involved in the differentiation and morphology of comb. To prove this further investigation of both of these genes is required.

For crest type, A highly significant genetic locus ($P<0.01$) was found on chromosome E22C19W28 at 7cM with an F-value of 120.9 (Chapter 5). The confidence interval spanned a region of around 13cM. A crest phenotype is characterised by tuft of elongated feathers at the top of head (Wang et al., 2012). A feather-crested head is a prominent feature exhibited by several wild bird species, as well as varieties of several domesticated birds (Bartels, 2003). The confidence interval of the QTL ranged from 4-17cM (13cM). Most of the genes in this confidence interval are related to keratin including KRT8 (Keratin 8), KRT4 (Keratin 4), Q6PVZ3_Chicke (type II alpha-keratin IIC) LOC426897 (similar to keratin), K2CO_Chick (Keratin type II), KRT80 (keratin 80). From this list of genes it seems that keratin is likely to be a potential candidate for the formation of crest. This assumption is supported by Ng et al. (2012) who concluded that alpha-keratins have an important role in establishing the structure of feathers. It's also found in the literature that crest is associated with an abnormality of skull known as cerebral hernia(Brothwell, 1979). Although in the present study we did not study cerebral hernia. Results of the current study are in consistent with Wang et al. (2012). They correlated crest phenotype with ectopic expression of HOXC8 in the cranial skin and mapped this on linkage group E22C19W28. They did not focus any Keratin gene present on this linkage group. In the current assembly, this gene is still mapped on unknown linkage group.

One highly significant QTL ($P<0.01$) for Silkie feathering was observed on chromosome 3 at 169cM. The length of C.I for significant QTL on chromosome 3 was 6cM (Chapter 5). Although there are limited number of genes and further dissection of these genes would give opportunity to study the biology of Silkie feathering. Due to its limited economic importance, we did not consider this trait for further fine mapping.

For feathered leg, initially, when data was analysed with microsatellite markers, three putative loci were found on chromosomes 5 ($F = 5.71$), 9 ($F = 6.97$) and 13 ($F = 6.81$) at 113cM, 47cM and 47cM (Chapter 5). After fine mapping, the loci on chromosome 5 and 9 disappeared on these chromosomes but the locus on chromosome 13 remained and its confidence interval decreased from 47cM to 20cM (Chapter 6). Although the C.I is reduced but still need to do further work to reduce the size. Current results are in accordance with the latter finding of Somes (1992) who studied different breeds for the feathered leg trait and concluded that Silkie feathered leg is caused by one or two incompletely dominant gene. This trait is interesting to study but if we see back the ancestors of domestic chicken, this trait is not present in any of the wild jungle fowl (Fumihito et al., 1994). This trait is supposed to have evolved by phenotypic mutation during animal husbandry and its functional importance never been considered in the earlier classical breeding experiments (Dorshorst et al., 2010). Xu et al. (2003) discovered a 124–128 million-year-old dromaeosaur fossil (Microraptor gui) in China having wing-like feathers on the legs. This extraordinary fossils could glide representing an intermediate stage towards the active, flapping-flight stage (Padian and Dial, 2005, Hone et al., 2010, Longrich, 2006) as opposed to the powered running theory on the origin of flight in

the ancestors of modern avian species (Prum, 2003). The feathers on the shanks of Microraptor gui may be similar to feathered shank as we see in any of the domesticated species.

Leg feathers are present in many fossil dromaeosaurs, early birds, and living raptors, and they play an important role in flight during catching and carrying prey (Chatterjee and Templin, 2007). Although the Confidence interval of the trait on chromosome 13 is still large but further strategy is required to reduce its size to reach the genes controlling the trait. These genes might be helpful to know the developmental mutant involved in the feathering of leg and also their role in sustaining flight in the ancient taxa.

For feather patterns, we found that the loci for barring, speckled and lacing remained on E22C19W28 ($P<0.01$) at a near identical position. From the present study it is concluded that other than the dominant white locus at the location of PMEL17, there is still some other genetic difference controlling the feather colour in chicken at this position. After removing White leghorn allele from the dominant white locus, there are still 8 white feathered individuals in F_2 population. It is also speculated that presence of mutations other than dominant white might be a cause of white feathering in the founder of Silkie birds and in some of white feathered individuals in F_2 population..

8.4 Conclusion

First time, a comprehensive study was conducted to cover the major genetic aspect of incubation behaviour. This study demonstrated the basis for genetic determination of incubation behaviour. Although comprehensive effort was done to explore the genetics of incubation behaviour but still there are certain short coming which need to fix by increasing resource population. As this is the major problem of some of the developing countries where tradition poultry is contributing a lot towards the national economy. These population could be a source for the provision of more recombination to limit the confidence interval and also helpful to reduce the shortcoming of the current study.

Current study also shed light on the genetics of various Morphological traits. It is also helpful to explore the quantitative nature of certain morphological traits (Feathered leg, Feather colour and pattern) which were initially thought to be Mendelian traits. This study opens opportunities for the researchers to study the developmental aspects of the traits and in the case of comb and crest we might offer alternative hypothesis to that posed by the recently published papers (Wang et al., 2012, Imsland et al., 2012) although the genetic loci are similar.

8.5 Limitations

1. Most of the QTLs regarding incubation behaviour are overdominant. In current study F2 population was used, there is also possibility that these QTL might segregate in one family rather than in whole population. To find out the linkage disequilibrium of QTL in a population there is need to test these

up to third or fourth generation. This was not possible with the current population as this population was not further reared due to high rearing and phenotyping cost.

2. It's also worthwhile to test effect of these QTLs in a new population before implication.

8.6 Future work

1. First time a comprehensive project was conducted to find the genetics of incubation behaviour. This study provides a sound base for further exploration of incubation behaviour by conducting genome wide association study in other population.
2. QTL for feather type found on chromosome 3 with confidence interval of 3cM also give a better opportunity of limited genes and further dissection of these genes give the exact mutation and also helpful to know the biology of Silkie feathering in the Silkie.
3. It is also found that WNT6 may be the potential candidate responsible for the genetic variation of comb. It is also proposed that this gene (Wnt6) could be knocked out or silenced in different chicken population to further validate its role in comb development.
4. Due to time constraints, all the morphological traits could not be explored up to gene and SNP level. But this study gives excellent opportunity for further dissection of the traits by sequencing the confidence intervals of all the found genetic loci.

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