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Fine Mapping of Autoimmune Diseases in Chickens

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ABSTRACT

Autoimmune diseases arise due to failure of the body's immune system to recognize its own tissues. Systemic sclerosis is a disease of the connective tissue characterized by immunological abnormalities, increased deposition of extracellular matrix proteins in the skin. Vitiligo is a dermatological disorder characterized by loss of melanocytes from the cutaneous epidermis. The aim of this project was to confirm the previously identified QTLs in F3-F5 and BC2 generations. We confirm the association of the disease with our marker genotypes on chromosome 3 but not on chromosomes 9 and 21 in the SL and BL crosses from F3-F5 generations. We did not confirm the disease association with chromosomes 2, 12, 14 and 19 in the BC_2 generation from the UCD x JF cross because the pedigree was not large enough for the analysis. 68% of the individuals in the BC_2 pedigree expressed the diseases with males showing 100% liability. We recorded a mortality rate of approximately 50% in the BC2 population. We did not observe any significant mortality in the inter-crosses between the SL and BL.

Keywords: chicken, autoimmune disease, animal models, systemic sclerosis, vitiligo

BACKGROUND

Chickens and humans shared the common ancestor approximately 310 million years ago (Hedges 2002, Hillier *et al.* 2004 and Burt 2005). The red jungle fowl (*Gallus gallus*) is considered to be the ancestor of all domestic chickens (West and Zou 1988, Fumihito *et al.* 1994, Wong 2001, Kanginakudru 2008). The chicken (*Gallus domesticus)* was domesticated over 8 000 years ago in South East Asia. It was originally used for religious purposes, leisure, cock fighting and the Romans used the chicken as oracles (Crawford 1996). It was later used as an agricultural animal with specialized breeds generated for different purposes. It is a source of protein (meat and eggs) with billions of eggs used each year (Burt 2005). Some chickens are kept as pets such as the Bantam and the Silkies since they are more docile (Clucks & Chooks 2008).

The chicken as an animal model

Animal models are important in providing clues for understanding human diseases. Evidence of the use of chicken as the animal model described by Aristotle was the use of chicken in embryology. Harvey (1628) studied blood circulation using chickens while Pasteur (1880) discovered transmission of infections in chickens through attenuating a virulent pathogen of chicken cholera. Today, the chicken is an important experimental system for developmental biology, immunology, microbiology, genetics, virology and cancer (Jimenez *et al.* 1984, Brown *et al*. 2003, International Chicken Genome Sequencing Consortium 2004 and Stern 2005). Animal models for understanding diseases were based on whether the diseases are induced by exogenous compounds or genetically inherited as a trait (Van de Water *et al*. 1995).

Experimental animal models that spontaneously develop the autoimmune disease reflect the human situation more closely than in models where the disease has been induced (Erf 2010). Even though murine models have been broadly used in biomedical research for autoimmune diseases they are very rare and currently there is no model that spontaneously develops autoimmune vitiligo (Erf 2010).

The chicken has several advantages as an animal model for studies of biological processes and they include a rich genetic diversity, large population size, ease of breeding, high rates of recombination (Kerje 2003, Siegel *et al.* 2006) and this has been enhanced by the construction of the chicken genome sequence and identification of 2.8 million SNPs (International Chicken Genome Sequencing Consortium 2004 and International Chicken Polymorphism Map Consortium 2004).

Chicken models of autoimmune diseases

Chicken model of systemic sclerosis

Complex pathophysiology of scleroderma/systemic sclerosis (SSc) has led to the development of animal models, which gives a closer look at pathophysiology of the disease such as functions of various cell types like monocytes, T-lymphocytes, fibroblasts and mast cells (Haustein 2002).

The University of California at Davies lines 200 and 206 (UCD 200 and 206) developed by Bernier in 1942 has become the ideal model for the study of SSc since it shows the entire manifestation of clinical, histopathological and serological spectrum of SSc while other animal models (tight skin mice) of SSc lack vascular damage (Sgonc 1999 and Wick *et al.* 2006). In this chicken model, F_1 generation show no incidence of the disease while F_2 generation had a disease incidence of 4%.

Other animal models of SSc

Other animal models of SSc include the tight skin mouse (*Tsk*). The *tsk* mouse has thickened skin that is tightly bound to the subcutis and deep muscular tissue. Compared to human scleroderma, the *tsk* mice lack dermal sclerosis and fibrosis is deeper than in humans but biochemical and molecular abnormalities similar to human SSc were noted (Yamamoto 2010).

In bleomycin-induced scleroderma, bleomycin (an isolate from fungus *Streptomyces verticulus*) is used. Bleomyin produces free radicals, induces apoptosis and exerts effects on skin-constituted cells such as fibroblasts, keratinocytes, endothelial cells and immunocytes (Yamamoto 2010).

In the fibrosis mouse model of TGF-β/CTGF, mice injected with TGF-β showed granulation and fibrosis of the skin (Yamamoto 2005). Transforming growth factor is a powerful stimulus for in-vitro formation of collagen. (Kanzler *et al*. 1999)

Chicken model of vitiligo

The Smyth line (SL), developed by Dr J Robert Smyth in the 1970s is well established as an animal model (Smyth 1980). SL chickens show post hatch loss of melanin synthesizing melanocytes in feather and choroid tissue. The SL chicken is the only animal model that mimics the entire spectrum of clinical and biological manifestations of the human disease (Wick *et al.* 2006).

Liability of vitiligo in the Smyth line is 70-95% within the contemporary group and de-pigmentation in the feathers is seen at 6-20 weeks of age with animal showing complete de-pigmentation in adulthood (Erf 2007). However, the parental Brown line (BL101) with matched major histocompatibility complex (MHC) has less than 2% incidence of vitiligo while the Light Brown (LBL101) is vitiligo resistant.

Incidences of the disease in this animal model are predictable and the lesions are easily accessibility since the feather tissue regenerates. Smyth (1989) characterized the disorder as spontaneous vitiligo-like loss of melanin producing cells in the feathers and the choroid after hatching.

Other animal models of vitiligo

Several species having naturally occurring de-pigmentation have been proposed as models for vitiligo. One example is *Grey* horses such as Lipizzaner, Arabian and Andalusian. These horses are normally coloured at birth and those carrying the dominant *Grey* allele show de-pigmentation after birth which increases with age until they appear completely white at maturity (Boissy and Lamoreux 1988), nevertheless a duplication of the STX17-region was denoted to be the causative mutation leading to premature loss of pigment and melanoma in these horses (Pielberg *et al*. 2008), and therefore this is not a good model for autoimmune as no autoimmune component is involved.

The Sinclair miniature swine model of vitiligo develops spontaneous regression of cutaneous malignant melanoma lesions (Hook *et al.* 1982). This pig is considered a good model for spontaneous melanoma. De-pigmentation is associated with the tumours together with development of uveitis and vitiligo and there is evidence of immune system involvement in tumour regression and development of vitiligo (Erf 2010). Vitiligo in the water buffalo was observed to resemble vitiligo in humans in cytological aberrations of melanocytes but no autoimmune component has been confirmed (Cerundolo *et al.* 2007).

The vitiligo mouse (C57B1/J6vit/vit) has in addition to progressive loss of hair and ocular pigmentation with age also contacts allergens as observed in human vitiligo (Boissy *et al.* 1987). However, the phenotype was mapped to the microphthalmia (MITF) locus ($mi^{vit/vit}$) (Lamoureux *et al.* 1992) and therefore this animal model was excluded as a model for autoimmune vitiligo.

The Barred Plymouth Rock and White Leghorn chicken lines were thought to be models of vitiligo (Bowers *et al.* 1992). In Barred Plymouth Rock chickens the black and white band patterns results from autophagocytic degeneration of melanocytes, which are not present in the white bands. The *barring* phenotype was lately linked to two non-coding mutations in the *CDKN2A* and two missense mutations in a highly conserved region (Hellström *et al.* 2010). The *barring* gene was found to inhibit melanin deposition in the feathers, beak, shanks and the eyes of the chicken (Erf 2010). The white feathers in the White Leghorns are known to be caused by a mutation in the *PMEL17* gene (Kerje *et al.* 2004). Due to the fact that all de-pigmentation phenotypes described above are caused by mutations in singe genes eliminates all of them as models for autoimmune vitiligo.

AUTOIMMUNE DISEASES

Autoimmune diseases arise as a result of failure of the body to recognize its own cells and tissues and allow the immune system to respond against them. The immune system creates autoantibodies against defective or abnormal cells that result from factors like inheritance and environment leading to inflammation and autoimmune disorder. Autoimmune disorders fall into two categories: systemic, which damages many organs and localized which attacks specific organs and tissues even though it can extend beyond the targeted tissue to other body organs (Ravel and Descotes 2004 and http://www.aacc.org). The precise cause of autoimmune diseases is generally unknown but it is suggested that there appears to be some genetic predisposition to develop autoimmune disease in many cases.

Systemic sclerosis

Systemic sclerosis is as a heterogeneous disorder that affects the connective tissue of the skin and internal organs such as the lungs, intestinal track, heart and kidney (Haustein 2002). It is characterized by vasomotor disturbances, fibrosis, subcutaneous and skin atrophy as well as muscle and internal organs arterial occlusion, polyarthritis and presence of antinuclear antibodies, peri-vascular mononuclear cell-infiltration and increased deposition of extra cellular matrix, microvascular damage, immune system disturbance and massive collagen deposition (Gershwin *et al*. 1981, Sgonc 1999, Yamamoto 2002, Jimenez and Derk 2004, Schwartz *et al.* 2009, Rabquer *et al.* 2009).

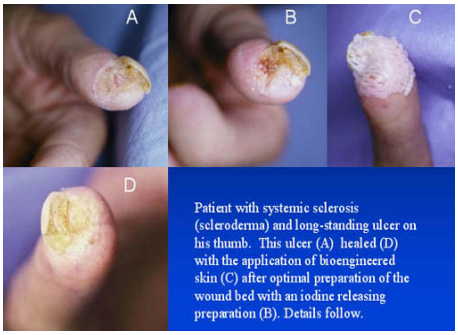
Aetiology of SSc is assumed to be immunologic mechanisms, genetic risk factors and environmental triggers as well as interaction between genetic risk factors and environmental triggers (Jin *et al*. 2007, Rula and Hajj-Ali 2008, Maron *et al.* 2010). In humans, familial clustering of SSc is rare (Van de Water and Gershwin 1985) and this might be because familial cases of the disease have not been well documented or environmental factors are very important and this can be proven in the UCD 200 and 206 lines since they have been heavily selected for the disease.

In humans SSc is characterized by fatigue, intoleranrance to cold, pain, deformity and loss of function of the hand as well as change in facial appearance (Medsger 1991). Pathohistological findings indicated that fibroblasts, endothelial cells and lymphocytes are key main players in three major clinical observations which are fibrosis of cutaneous and viscera, vascular alterations and immunological alterations (Wick *et al* 2006, Yamamoto 2005, 2009). Progressive thickening and fibrosis of the skin is usually noted and in subclinical cases internal organs are affected (Fleischmajer *et al.* 1977, Haustein 2002 and Wick *et al.* 2006). In humans SSc starts with oedema of the fingers preceded by Raynaud's phenomenon (Yamamoto 2009). Fibrosis can be caused by profibrotic cytokines including transforming growth factor beta (TGF-beta), interleukin 4, platelet derived growth factor (PDGF) and TGF-beta (http://www.aacc.org).

In the UCD 200 chickens, SSc is characterized by swelling, erytheroma and necrosis of the comb (self dubbing) (Fig 1) (Van de Water and Gershwin 1985). Van de Water and Gershwin (1985) also observed swelling of the peripheral joints as well as polyarthritis, swelling of the upper neck sac after 4 weeks of age. Other visceral organs involved include oesophagus (64%), small intestine (60%), lung (60%), kidney (65%), heart (30%) and testis (66%). Antinuclear-antibodies have been detected in 65% of the UCD 200 chickens at 6 months of age, which also produced rheumatoid factor at 4 months were noted (Van de Water and Gershwin 1985).

A comparative study of skin biopsies from UCD 200 chickens and biopsies from human SSc patients revealed that endothelial cell apoptosis induced by anti-endothelial cell antibody (AECA) - dependent cellular cytotoxicity is a principal event in the pathogenesis of SSc (Sgonc 1999). Histopathological findings of the skin involve inflammatory infiltrates of lymphocytes, monocytes, plasma, histiocytes around the blood vessels and sweat glands. Infiltrating immunocytes releases fibrogenic cytokines, which play a very crucial role in the initiating events in SSc (Jimmenez *et al.* 1986). Comparative details of SSc in both humans and chickens are described in Table 1.

Fig 1. UCD 200 chickens showing swelling and necrosis of the comb and feet necrosis (Photos. Wick et al. 2006)



http://www.goldbamboo.com/pictures-t7460.html

Fig 2. Systemic sclerosis patients showing polyarthritis in fingers equivalent to feet necrosis in chickens.

Several vitamins, pharmaceuticals, hormones, drugs and surgical procedures are reported to be successful in the traetment of systemic sclerosis and this treatment have been well accepted (Medsger 1991).

Table 1. Comparison of clinical, biochemical, immunological, and pathologic features of human and avian SSc, source Wick et al. 2006.

	Human SSc	**Avian SSc**
Clinical features		
Disease presentation	Subtle, middle age	Acute, early in life
Skin fibrosis	Present	Present
Esophageal fibrosis	Present	Present
Lung fibrosis	Present	Present
Kidney involvement	Present	Present
Heart involvement	Present	Present
Polyarthritis	Present	Present
Eye involvement	Debated	Absent
Autoimmune features		
Autoantibodies		
ANA	Present	Present
Anti-Scl-70	Present	Absent
Anticentromere	Present	Present
Anticytoplasmic	Present	Present
Autoreactive T cells	Present	Present
Aetiology		
Genetic susceptibility	Necessary not sufficient	Necessary and sufficient
Environmental factors	Hypothesized	Not important
Pathology		
Fibroblast alterations	Present	Present
Endothelial alterations	Present	Present
Smooth muscle alterations	Present	Present

Cytokines and chemokines (receptors) in scleroderma

Cytokines are a large family of glycoproteins that regulates fundamental biological processes, which includes immunity, wound healing, haematopoiesis and development of the nervous system and are an essential component of adaptive and innate immune systems (Krebs and Hilton 2001, Alexander and Hilton 2003). Chemokines are a small family of cytokines that participate in immune and inflammatory responses through chemo-attraction and activation of leukocytes (Baggiolini 1998). Chemokines function as leukocyte activators and chemo-attractants in inflammatory responses (Luster 1998). In aetiology of scleroderma, there is growing evidence that overproduction of extracellular matrix by activated fibroblasts results from the interaction between endothelial cells, macrophages, lymphocytes and fibroblasts through mediators such as cytokines, chemokines and growth factors (Yamamoto 2009).

Suppressor of cytokines signalling 1 (SOCS 1)

The suppressor of cytokine signalling 1 is a key negative regulator of cytokine signalling and immune response (Egan *et al.* 2003, Hanada *et al.* 2003, Fenner *et al.* 2006). The SOCS1 family of proteins forms part of the negative feedback system that regulates cytokine signalling transduction by binding to the cell surface receptors and activating intracellular signal transduction such as the JAK-STAT pathway (Krebs and Hilton 2001, Alexander and Hilton 2004). The SOCS1 protein target signal transducers for proteosomal destruction (Alexander and Hilton 2003) and expression can be induced by a subset of cytokines IL1, IL3, erythropoietin and interferon gamma (Hanada *et al*. 2003).

Chemokine ligand 1 (CCL1) and chemokine receptor 8 (CCR8)

CCL1 is a small glycoprotein secreted by activated T cells belonging to a family of chemokines. CCL1 attracts monocytes, NK cells, immature B cells and dendritic cells by interacting with a cell surface of chemokine receptors, CCR8 (Millner and Krangel 1992, Roos *et. al* 1997, Hapel and Haque 2002, Haque *et al.* 2004). CCR8 is a G-protein-coupled 7 transmembrane receptor that is unique for the CCL1 and viral monocyte inflammatory protein-1. It was found to be a functional endothelial receptor mediating endothelial chemotaxis in response to CCL1 and vCCL1 (Dairaghi *et al*. 1999).

Other cytokines involved in scleroderma include the transforming growth factor beta (TGF-β), released by activated macrophages or lymphocytes, chemically attracts fibroblasts and increases the synthesis of extracellular mass e.g. collagen type I and type III and fibronectin (Yamamoto 2009). TGF-β increases levels of TGF-β receptors in fibroblasts leading to increased TGF-β production and hence high deposition of extracellular mass and fibrosis (Yamamoto 2009).

Connective tissue growth factor (CTGF) is induced in fibroblasts after activation by TGF-β (Frazier *et al.* 1996, Yamamoto 2009). CTGF protein stimulates DNA synthesis and up-regulates collagen, fibronectin and integrin expression in fibroblasts (Frazier *et al.* 1996).

An existing disproportion between type I and II cytokine responses have been reported in the pathogenesis of scleroderma. Interleukin 13 has the capability to suppress pro-inflammatory cytokines production in monocytes and macrophages and enhance the growth and differentiation of B cells and promote immunoglobulin synthesis (Yamamoto 2009).

Pro-inflammatory chemokines are associated with the initiation of fibrosis and sclerosis. This led to the suggestion that chemokines and chemokine receptors may have a critical role as mediators of inflammation and fibrosis (Yamamoto 2009).

Autoimmune vitiligo

Vitiligo is an acquired dermatological disorder characterized by loss of epidermal melanocyte leading to de-pigmentation of the skin (Wang and Erf 2004). It is thought that vitiligo is due to a combination of several factors which, include genetic susceptibility, immune system and environmental components including several kinds of stress (emotional) accumulation of toxic melanin precursor in melanocyte (DOPA, dopachrome, 5 6-dihydroxyindole) and disturbance of melanocyte homeostasis (impaired intra and extra cellular calcium) (Wick *et al.* 2006 and Kakourou 2009).

The disease is known to affect up to 2% of the world population (Erf *et al.* 2001, Namazi 2005, Wick *et al*. 2006 and http://www.aocd.org). Vitiligo is often associated with other autoimmune diseases such as hypothyroidism (4-8%), alopecia areata (2-3%) and 5- 15% uveitis often resulting in blindness. People with vitiligo are more prone to develop Addison's disease, pernicious anaemia and diabetes mellitus, type I (Erf 2010).

The disorder usually appears as focal pattern where the disease is limited to one or few areas, segmental pattern where de-pigmentation occurs only in one side of the body and as generalized pattern which is the most common and occurs symmetrically in both parts of the body.

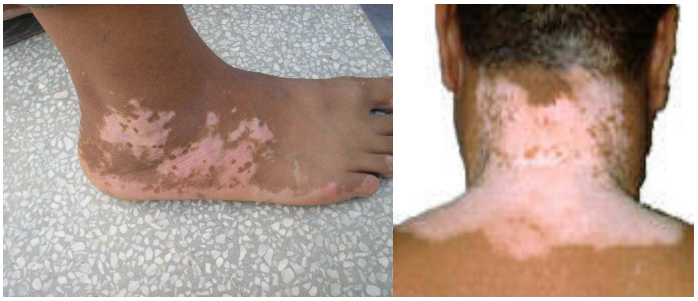


Fig 2. Patients with vitiligo showing loss of pigmentation.
Photos (http://www.psoriasiscafe.org/vitiligo-pictures.htm)

In both chickens and humans, vitiligo is characterized by spontaneous loss of pigmentation in association with uveitis and underlying hair as well as oral mucosa (Lamont *et al.* 1982 and Alkhateeb *et al.* 2003). There is an increased frequency of association of vitiligo and several antibodies suggesting hyperactive immune system in humans and in some patients autoantibodies are directed against melanocytes or melanocyte proteins (Lamont *et al.* 1982 Alkhateeb *et al.* 2003). Evidence of strong association with anti-melanocyte antibodies in both animals and humans has been reported (Norrirs *et al.* 1988). An intrinsic heritable melanocyte defects with abnormal and irregular surfaces predisposes the SL chickens to the pigment disorder (Boissy *et al.* 1983).
The immunopathology of Smyth line vitiligo has been shown to support the involvement of cell mediated immune response in destruction of the melanocytes causing white feathers in the chickens (Fig 2) (Wang and Erf 2003, Wang *et al.* 2004). The environmental component

in the expression of autoimmune vitiligo was shown by vaccination of chickens with a live Turkey herpes virus vaccine (HVT), which triggered the expression of SL vitiligo (Erf *et al.* 2003). HVT show a strong tropism for epithelium of feather follicles, however some studies have revealed that killed HVT had no effect in the expression of vitiligo (Erf et al. 2001, 2003). Vaccinations with Newcastle disease virus (NDV) and Infectious bronchitis virus (IBV) did not trigger vitiligo suggesting that viral infections and antiviral immune activity are not responsible for the expression of autoimmune vitiligo (Erf 2010).

Fig 3. Phenotypes in SL x BL intercross chickens with vitiligo (Photos. S. Kerje).

Vitiligo in humans starts with irregularly shaped melanosomes containing pigmented membrane extensions, hyperactive melanization and selective autophagocytosis of melanosomes. Both of the aspects of immunity, humoral and cellular, have been implicated to have a role in the pathogenesis of vitiligo (Boissy *et al.* 1984). In the SL chickens, vitiligo also starts with the defect in melanocytes followed by an autoimmune response, which involves humoral and cellular reactions that eliminate abnormal pigment cells (Rezaei *et al.* 2007). The neural theory is associated with segmental vitiligo while the autoimmune hypothesis is thought to be associated to generalised vitiligo (Shajil *et al.* 2006).

Tissue destruction by the autoimmune cells in humans and animals is associated with lack of regulatory function within the immune system, hyperactive immune response and altered immune response to endogenous and exogenous factors (Erf 2010).
It has been shown that the presence of retroviral genes is linked to the expression of vitiligo in the SL chicken model and this is because of the structural and sequence similarity to exogenous viruses linked to immune dysregulation and their tissue specific expression (Sreekumar *et al* 2000).

Increased T cells in the feather pulp of the SL chickens have been noted together with the inflammatory leukocytes before and during the onset of vitiligo (Erf 2010). In all (100%) of the SL chickens, antibodies against melanocytes have been detected in serum (Rezaei *et al* 2007). Melanocyte death occurs by apoptosis and is induced by cytotoxic T cells (Erf *et al* 2001 and Wang *et al.* 2004). In the melanocytes of mammals the target antigen for the antibodies is the tyrosinase related protein (TRP-1). Anti-pigment cell antibodies, which recognize the same melanocytes antigens as in humans have been identified in horses, cats and dogs.

Treatment

Treatment and re-pigmentation of vitiligo in patients can be achieved with immunosuppressive agents such as ultraviolet radiation in combination with Psolaren (PUVA), corticosteroids and cytotoxic drugs.

THE CHICKEN GENOME

The chicken genome sequence has enhanced the chicken as an animal model (Edwards *et al.* 2005). Analyses have shown that chickens and humans share about 60 percent of their genes whereas 88 percent is shared between humans and rodents. This has made orthology relationships between chicken and humans more complex and non detectable because some proteins and domain families are under-represented in the chicken as compared with mammals. The chicken genome, however, provide new insights on evolution, structure and gene content of mammalian genomes (International Chicken Genome Sequencing Consortium 2004).

The chicken genome is 1.05×10^6 bp (International Chicken Genome Sequencing Consortium 2004) divided into 39 pairs of chromosomes (9 macrochromosomes and 30 microchromosomes). Microchromosomes have higher G+C content, more CpG islands as well as high gene density, shorter introns, few repetitive sequences and high recombination rates. The female is the heterogametic sex carrying Z and W chromosomes while males are homogametic ZZ (Burt 2007).

The chicken genome is estimated to have 20 - 30 000 genes and approximately 30-40% of the mammalian genome in size. The difference in size is attributed to reduction of interspersed repeats, segmental duplications and pseudogenes of which the chicken has 10% as compared to 50% in humans (International Chicken Genome Sequencing Consortium 2004, Burt 2007). Only 44% of the conserved sequences represent protein coding genes while conserved non-coding sequences are located far from well-defined genes and may have important regulatory functions (International Chicken Genome Sequencing Consortium 2004).

2.8 million SNPs have been reported in the chicken genome database (International Chicken Polymorphism Map Consortium 2004, International Chicken Genome Sequencing Consortium 2004, Wong *et al.* 2004). Aligned segments of the chicken and human genomes occur in long syntenic blocks with low rates of translocations and more frequent intra-chromomosomal rearrangement from the last common ancestor (International Chicken Genome Sequencing Consortium 2004).

MAPPING STRATEGIES

Quantitative Trait Loci (QTL) mapping is described as the application of techniques to identify the genes controlling a certain trait. Identification of QTLs is critical for understanding the basis of complex traits (Broman 2001). Mapping complex traits has more challenges since it is not possible to follow all genomic regions underlying complex traits without knowledge of how they segregate (Doerge 2002). Even if statistical associations between QTL and markers have been found, genomic regions are sometimes large and expensive to undertake and there are also some genetic interactions such as epistasis and non-genetic factors to take into account (Doerge 2002, Zou 2009).

According to Broman (2001) experimental populations need to be fixed for relevant traits and this is achieved by repeated mating of siblings. A series of crosses is necessary of which backcross design is the simplest. Usually, an F_2 generation is used and the most favourable situation in an F_2 design is when all genes underlying the trait of interest are diallelic and alternative alleles fixed in both parental lines (Haley and Knott 1994, Perez and Varona 2000, Andesson 2001). QTL experiments can be designed in a way that enable analysis of complex phenotypes be achievable and this requires large sample of individuals that represent the whole population and provide a number of observable recombinations (Doerge 2001).

Two approaches have been assumed in QTL mapping studies and they are the infinitesimal, which assumes that traits are determined by the unlimited number of genes, each with a small effect, and the finite model which presumes a restricted or finite number of genes with a major effect (Hayes 2008). Two common methodologies for QTL mapping include the candidate gene approach (assumes that a gene could harbour a mutation that results in genetic or physiological variation of a trait) and linkage mapping which uses recombination and DNA markers to construct a linkage map (Hayes 2008).

Linkage Mapping

Linkage mapping is determining the order of genes or markers on a chromosome and the relative distance between those genes based on the relative recombination frequencies. Genetic maps are essential tools for mapping and dissection of complex quantitative trait loci and allow for systematic genome scan for genes affecting all kinds of traits (Miles *et al.* 2009).

Linkage mapping is very essential in the identification of candidate genes that may not have been studied in details as it allows defining of chromosomal regions that harbour the genes affecting the phenotype (Wick *et al.* 2006). Wick *et al.* (2006) described linkage mapping as one way of excluding candidate genes since it discriminates genes not in linkage with the trait locus, which is better than by functional studies. Linkage analysis looks for regions in the genome with a higher proportion of shared alleles among affected individuals, which depicts that there is a disease predisposing allele (Carlson *et al.* 2004). However, linkage has not worked well when trying to identify the genes underlying complex diseases (Reich 2006).

PRELIMINARY RESULTS

In a previous study (Ek *et al.* unpublished), a backcross generated after crossing pure line UCD 200 birds with F_1 birds from a cross between UCD200 and red jungle fowl was used to identify the genes underlying comb necrosis in the UCD 200 line. A whole genome scan including 384 SNPs uncovered 4 genomic regions associated with comb necrosis on chromosome 2, 12, 14 and 19 (Fig 4).

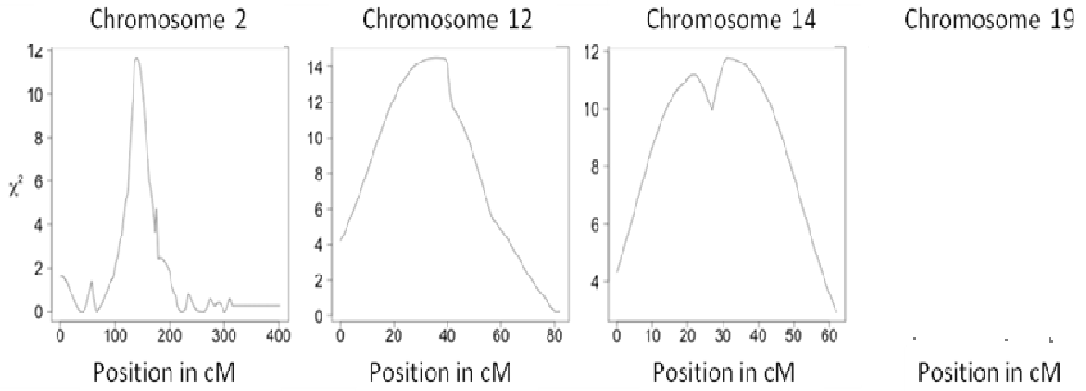


Fig 4. QTL regions identified in the backcross generation after matings between F_1 and pure line UCD 200. Chromosomes 2 and 14 were highly significant whereas 12 and 19 were suggestive. QTLs on chromosome 2 and 12 were identified for comb necrosis at 21 days of age while chromosomes 2, 14 and a suggestive QTL on 19 were identified for comb necrosis at 175 days of age.

In the autoimmune vitiligo animal model a Hidden Markov algorithm was used to estimate the QTL genotypes since it was more efficient than gridQTL in handling markers with mixed information content (Ek *et al.* unpublished). The whole pedigree was genotyped with 384 SNP markers. Genome wide QTL scan of individual QTL effects using Haley-Knott regression and Flexible Intercross Analysis (FIA) revealed marker association on chromosomes 3, 9 and 21 with the vitiligo phenotype (Fig 5).

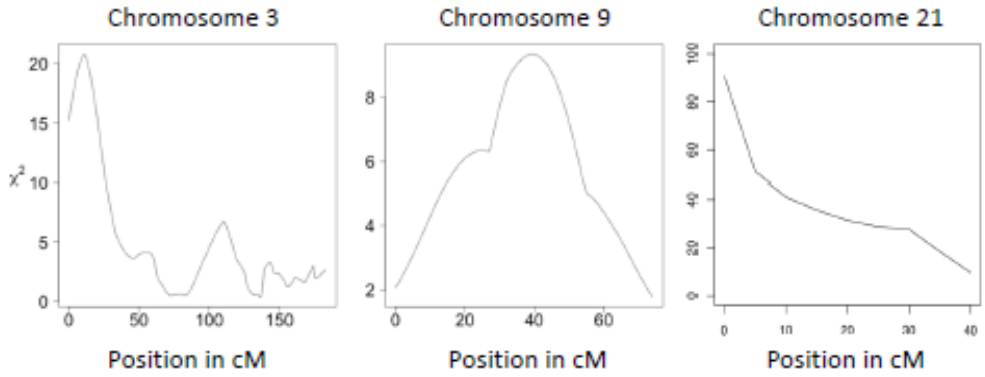


Figure 5. QTL regions implicated in autoimmune vitiligo in the SL chicken. Chromosome 3 was highly significant ($P < 0.0001$) in both Haley-Knott regression and FIA whilst chromosome 9 was significant in FIA and suggestive using Haley-Knott regression and chromosome 21 was suggestive ($P < 0.2$) in FIA.

The purpose of this project is to

- Confirm the QTLs for autoimmune vitiligo in the F_3, F_4 and F_5 intercross generations in a cross between SL and BL.
- Validate QTL association with comb necrosis in the 2nd backcross generation in a pedigree based on a cross between UCD 200 and red jungle fowl.
- Indentify SNPs in the candidate genes, SOCS1, CCR8 and CCL1 underlying the already identified QTLs for comb necrosis on chromosome 2, 12, 14 and 19 through sequencing of the UCD 200 and red jungle fowl parents from the cross.

MATERIALS AND METHODS

Animals

UCD 200 pedigree
8 red jungle fowls (4 males and 4 females) and 8 UCD 200 individuals (4 males and 4 females) were mated to produce an F_1 generation. 12 UCD 200 (4 males and 8 females) were crossed with F_1 generation birds to generate a large backcross pedigree of 598 individuals. BC_1 males were bred to UCD 200 females to produce 38 BC_2 individuals hatched in 3 batches.

Smyth line pedigree
14 Smyth line (SL) and 12 Brown line (BL) parental chickens were crossed to produce 57 F_1 individuals. F_1 were intercrossed to generate 496 F_2 individuals hatched in 7 batches. F_2 was subsequently intercrossed to produce 45 F_3 individuals which were intercrossed to produce 102 F_4 individuals hatched in 3 batches and F_4 were further intercrossed to generate 90 F_5 individuals hatched in 3 batches.

Phenotyping

All individuals in the BC_2 generation were visually observed and scored as affected, indicating disease, or non-affected for comb necrosis at adult age (more than 175 days of age). Individuals with comb necrosis have red, swollen and inflamed combs with part of the comb missing. Individuals scored as non-affected has no inflammation and a normally formed comb (Fig 1).

The vitiligo phenotype were recorded as binary traits, affected or non-affected. Affected individuals have lost pigmentation resulting in whole or partly white feathers. Non-affected individuals have a fully pigmented plumage. All individuals in the F_3 to F_5 generations were visually observed and scored according to the above mentioned criteria (Fig 3).

Blood sampling and DNA extraction

A total of about 2 ml blood was collected from the brachial vein for serum, plasma and whole blood with 5.5% EDTA solution as anticoagulant. Samples were collected from 19 individuals from the 2nd backcross (F1 x UCD 200) chickens at adult age and from 90 individuals from F_5 intercross generation from the SL x BL cross at 9 weeks of age.

DNA was extracted by the salt precipitation method using ethanol described by Miller *et al.* (1988) and dissolved in 100 µl 1 x TE solution (10 mM Tris pH 8.0, 0.1 mM EDTA). 10 µl of blood was used for DNA extraction as all blood cells are nucleated in birds. The amount and quality of DNA was measured using the Nanodrop ND 1000 Spectrophotometer (Saveen Werner AB, Malmö, Sweden) and the DNA was diluted to a concentration of 50 ng/µl.

Sequencing for detection SNPs in potential candidate genes

Primers were designed to amplify all exons and introns of the *CCR8*, *CCL1* and *SOCS1* candidate genes. Reference sequences were retrieved from the UCSC genome browser and the chicken reference genome sequence WUGSC 2.1/galGal3, 2006 (http://genome.ucsc.edu)

Gene Fisher software (Giegerich *et al.* 1996) version 2.0 was used for primer design. The sizes of the fragments amplified in the PCR are 781 bp for *SOCS1*, 1827 bp for *CCL1* and 3350 bp for *CCR8*. Internal primers were designed for *CCR8* and *CCL1* regions as sequencing from the ends does not cover the entire fragment. All primers can be found in the Appendix Table 8)

Amplification of the genes using parental DNA as template was carried out in Applied Biosystems 2720 thermal cyclers (Applied Biosystems, Foster City, California, USA). Hot StarTaq polymerase (Qiagen, Hilden, Germany) with a touchdown PCR temperature cycling was used to allow optimal amplification. The total volume of the PCR reaction was 10 µl including 1 x PCR buffer (Qiagen), 2.5 mM $MgCl_2$, 200 µM of dNTPs, 20 pmol of forward and reverse primer, 0.1 units of Hot StarTaq polymerase (Qiagen) and about 50 ng of DNA. The thermocylcing was as carried out as follows, initial activation of the polymerase at 94°C for 10 minutes, 14 cycles of denaturing at 94°C for 30 sec, annealing temperature starting at 65°C decreasing 1 degree per cycle to 52°C for 30 sec, extension at 72°C for 30 sec, followed by 26 cycles of denaturing at 94°C for 30 sec, annealing temperature of 52°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 5 minutes.

DNA fragments including *CCL1* and *SOCS1* genes were separated in a 2% agarose gel and fragments including *CCR8* was separated in 1% agarose gel in 0.5 x TBE (TrisBorateEDTA) and visualised under ultraviolet light after staining in an EtBr bath.

The QIAQuick Gel extraction Kit (Qiagen) was used for extraction of DNA fragments containing the amplified genes form the agarose gel. Elusion was done with 40 µl of elusion buffer (Qiagen) for fragments containing CCL1 and SOCS1 and with water for fragments containing *CCR8*. Sequencing of the amplified products was done at the Rudbeck laboratories in Uppsala.

CodonCode Aligner software, Version 3.5.6 (CodonCode Corporation, Dedham, MA, USA) was used to edit, align and compare the DNA sequences.

Genotyping using pyrosequencing

The DNA fragments used as template for pyrosequecnig were amplified using 3 primers. One primer was designed to anneal just in front of the SNP position and the other a bit further away containing an added tail to fit with the tail primer carrying the biotin label. The resulting amplified fragment contains a biotin that is used to fish out the fragment prior to pyrosequencing using the PyroMark Q96 machine (Qiagen) (See Appendix Fig. 5)

The SNPs selected for genotyping were previously identified to be informative in the cross and genotyped in all individuals in the pedigree.

PCR for amplification of SNPs genotyped in the UCD x JF BC_2 generation

Amplification of the SNPs that were genotyped was carried out with HotStarTaq polymerase protocol (Qiagen). The PCR reaction was carried out in a total volume of 25 µl containing of 1 x PCR buffer (Qiagen), 2.5 mM $MgCl_2$, 20 pmol dNTPs, 20 pmol of each primer except the tailed primer that had a concentration of 2 pmol, 0.1 units of HotStarTaq polymerase (Qiagen) and about 50 ng of DNA. The thermocycling was carried out as follows, initial activation of the polymerase at 94°C for 10 minutes followed by 14 cycles of denaturing at 94°C for 30 sec, annealing temperature starting at 65°C decreasing 1 degree per cycle to 52°C for 30 sec, extension at 72°C for 30 sec, followed by 26 cycles of denaturing at 94°C for 30 sec, annealing temperature of 52°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 5 minutes.

PCR for amplification of SNPs genotyped in the SL x BL F_3-F_5 generations

SNPs were amplified using Ampli-TaqGold polymerase (Applied Biosystems). PCR reaction was prepared to 25 µl including the reagents below; 1 x PCR buffer (Applied Biosystems), 2.5 mM $MgCl_2$, 20 pmol dNTPs, 20 pmol of each primer except the tailed primer that had a concentration of 2 pmol, 0.1 units of Ampli-TaqGold polymerase (Applied Biosystems) and about 50 ng of DNA. The thermocycling was carried out as follows, initial activation of the polymerase at 94°C for 4 minutes followed by 14 cycles of denaturing at 94°C for 30 sec, annealing temperature starting at 65°C decreasing 1 degree per cycle to 52°C for 30 sec, extension at 72°C for 30 sec, followed by 26 cycles of denaturing at 94°C for 30 sec, annealing temperature of 52°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 5 minutes.

Primer sequences for all markers genotyped can be found in Appendix Table 9.

Inheritance check was done manual to verify whether the alleles in the individuals are in concordance with their parental alleles.

STATISTICAL ANALYSIS

Single Marker Analysis

Genotypes of SL x BL intercrosses in the F_3-F_5 generations were analysed using generalized linear model in R software. This study used a binomial trait, that is either affected or unaffected, and therefore a binomial distribution was assumed. Generalized linear models are usually used for the analysis of binomial distributions. The ANOVA method is used when there are more than two marker genotypes.
The following model was assumed

y = sex + batch + ad + d + e

where ad = additive effects d = dominance and e = interaction

RESULTS

Table 2. Incidence of comb necrosis in the UCD 200 2nd back cross generation.

Sex	No. of affected		No. of unnaffected		Frequency of affected
	21 days	175 days	21 days	175 days	
Males		8		0	100
Females		5		6	42
Total		13		6	68

Table 3. Incidence of vitiligo in the SL x BL intercross, F3-F5 generation.

Sex		No. of affected		No. of unaffected		Frequency of affected
	F_3	F_4	F_5	F_3	F_4	F_5
Males	10	13	7	15	31	38
Females	6	6	3	13	37	38
Total	16	19	10	28	68	76
	36	22	13	64	78	87

SNP identification in candidate genes for systemic sclerosis

Comparison of sequences from JF 848, JF875, *SOCS1* and *CCL1* did not show any presence of SNPs.
We could only obtain a partial sequence from *CCR8.*

Pyrosequencing

Genotype data were obtained for all 7 markers genotyped. Only 2 individuals failed to give genotypes for marker RS13673471 for Chromosome 2.

QTL confirmation in the SLxBL F3-5 generations

The analysis gave suggestive evidence (P=0.1) for association between the marker located on chromosome 3, Table 4. No evidence of association was detected for markers on chromosome 9 and 21 (Table 5 and 6).

Table 4. Analysis of variance for chromosome 3.

Coefficients				
	Estimate std.	Error	z value	Pr(>|z|)
Intercept	-1.5254	0.4690	3.252	0.00114 **
sexM	0.3860	0.4156	0.929	0.35307
batch21	0.7285	0.4205	1.733	0.08318
ad	0.6163	0.3656	1.686	0.09187
dom	0.2071	0.5994	-0.346	0.72969

Signif. codes	0 '***'	0.001 '**'	0.01 '*	0.1 ' ' 1

** Z-values are used to compare samples to standard normal distributions (with $\mu = 0$ $\sigma = 1$). Error is deviation from normal values.*

Table 5. Analysis of variance for chromosome 9.

Coefficients				
	Estimate std.	Error	z value	Pr(>|z|)
Intercept	-1.0431	0.7341	-1.421	0.1553
sexM	0.3939	0.4120	0.956	0.3390
batch21	0.7433	0.4164	1.785	0.0743
ad	0.2215	0.4930	0.449	0.6532
dom	-0.9583	1,3060	-0.734	0.4631

Signif. codes	0 '***'	0.001 '**'	0.01 '*	0.1 ' ' 1

Table 6. Analysis of variance for chromosome 21.

Coefficients				
	Estimate std.	Error	z value	Pr(>|z|)
Intercept	-1.0463	1.7525	-0.597	0.5505
sexM	0.4076	0.4095	0.995	0.3196
batch21	0.7250	0.4129	1.756	0.0791
ad	-0.5736	0.9077	-0.632	0.5274
dom	-0.9086	3.3648	-0.270	0.7871

Signif. codes	0 '***'	0.001 '**'	0.01 '*	0.1 ' ' 1

DISCUSSION

A backcross pedigree structure (BC) was created by crossing the F_1 males from JF x UCD 200 cross to UCD 200 to increase the incidence of the disease from 4% observed in the F_2 to 50% in a backcross pedigree structure. Expression of comb necrosis indicating systemic sclerosis is alleged to be non-specific to any sex (Gershwin *et al.* 1981). This study shows that 100% of males in the 2nd backcross generation showed comb necrosis at day 200 while females showed an incidence of 42% making an overall liability of the 2nd backcross individuals to 68%. This frequency is higher in comparison to the frequency reported by Ek *et al.* (unpublished) were 95% of the males and 27% of the females in the first backcross (BC) generation were found to have comb necrosis and a study by Gershwin *et al.* (1981) who reported 95% of comb necrosis in all chickens. One possible explanation for this is that the BC_2 generation used in the study was small and perhaps not representative and phenotyping of the chickens may have been carried out at different ages. Another reason could be that the BC_2 chickens are old and the ones that are still alive are less sick, this may also explain the difference in frequency between the sexes, possibly females are more sensitive or maybe there are about unequal number of males and females still alive.

SSc in chickens can also appear in several forms ranging from limited skin involvement to severe or diffuse cutaneous (LeRoy *et a.l* 1988). In most cases fibrosis and thickening of the skin is observed while in some cases involvement can be confined to the digits (Fritzler and Kinsella, 1980) (Fig 2). Mortality in the UCD 200 is 20% at one month, 40% at 4 months and 55% at 5 months of age (Gershwin *et al.* 1981). We observed slightly equivalent mortality rates (49%) at 6 months (200 days) in our BC_2 individuals. Most of these animals died at less than 1 week of age precisely 1-3 days post hatch. The precise causes of these deaths are unknown since we did not carry out any tests.

Sequencing of the *CCR8* gene only provided partial sequence (from both UCD 200 and JF) This may be explained by the possibility that the DNA from the red jungle fowl parentals might be degraded and the gene is relatively large, 3350 kb, and therefore it is difficult to amplify a fragment of that length. One possibility could be that there may be some SNPs in the primer site that may cause inefficient annealing as low amount of the fragment could be amplified. Five SNPs can be found in the *CCR8* gene in the draft chicken sequence. They are positioned at 2:42719138, 2:42719308, 2:42719563, 2:42719645, 2:42720032 and the ones at positions 2:42719308 and 2:42719563 bp are synonymous A/G (rs15966955) and C/G (rs13671498) while non-synonymous SNPs were at positions 2:42719645, G/A (rs14171803) and 2:42720032 C/T (rs15966952) and a deletion G/- at position 2:42719138 (rs15090893) .

Comparing these SNPs: in a G/- deletion there is G/G in RJF genotype, for SNP C/T RJF has C/C genotype, for SNP C/G RJF has genotypes G/G while layers have A/A, in a C/G SNP broilers have genotypes G/G, RJF has C/C and the Silkie has G/G. For A/G SNP RJF has genotypes A/A while the Silkie has G/G. (See appendix, Fig.6 for comparative details)

In the synonymous variations, amino acids remain at leucine for C/T switch and histidine for G/A transition while in non-synonymous variations the amino acids in C/G switch change from glycine to alanine whilst in A/G shift leucine is changed to proline. The roles that these SNPs may have in the candidate genes are shown in Appendix Fig 7.

In the autoimmune vitiligo animal model, we noted frequencies of 16% in males and 7% in females of the F_5 generation. This is lower than expected because previous analysis showed a prevalence of 40% in the F2 generation. Nonetheless, we are still expecting more chickens to show the disease as they grow. It is known that the disease is more prevalent in the adolescence stage up to early adulthood (Erf 2010). We realized a reduction, in incidences of the disease from generation F3 to F5 at 21 weeks of age however, this should be verified as the chickens grow.

F_5 generation may not have been at the right time and more individuals may get the disease at a later stage.
We used one marker per QTL region in the statistical analysis. Since the analysis program used required a haplotype, i.e two markers, the genotyping marker was duplicated in the analysis. We did not observe any significant association in any of the 3 chromosomes analysed, only a suggestive association was found between the phenotype and the marker on chromosome 3. These results were not surprising because in the previous generation analyzed, the QTL on chromosome 3 was significant in both Haley-Knott and FIA ($P < 0.0001$) whereas the QTL on chromosome 9 was only significant in FIA ($P < 0.05$) and the one on chromosome 21 was suggestive ($P < 0.2$) (Ek *et al.* unpublished). Simulations and analyses of experimental data showed that the power of FIA was equal or better than Haley-Knott regression and insensitive to allele fixation in the founders. This method provides good estimates of QTL as well (Rönnegård *et al.* 2008).

Genotyping families with additional markers may increase the information content hence improving the likelihood of detecting true susceptible loci. Results from simulation studies suggested that breeding populations in QTL mapping should be more or equal to 100 individuals (Darvasi and Soller 1995). Our study populations for F_3 and F_4 are lower than the suggested size except for F_5, and this may lower the power of mapping. Rönnegård *et al.* (2008) showed that using few parental lines to generate a large F_2 population increases the power of QTL detection while using a large parental population increase the chance of having more alleles with different effects if they are not fixed.

FUTURE PROSPECTS

A large population of UCDx JF BC_2 individuals should be created to enable and increase the power of the analysis, did you do an analysis of these?

In the SLxBL F5 generation several more markers should be genotyped under the QTLs and males recombinant for different regions should be selected and crossed with pure line SL females to generate a back-cross to narrow down the QTL region. Darvasi and Soller (1995) showed that advanced intercross lines provide a 3 to 5 fold reduction in the confidence interval of QTL map location after 8 generations i.e. in F_{10} as compared to an F_2 or a backcross.

When potential candidate genes have been identified, their functions can be studied using the tissues that have been collected and are currently stored in freezers. Expression analysis could be carried out after extraction of RNA from the collected tissues (See list of collected tissues in Appendix Table 3).

CONCLUSION

Generally, causes of autoimmune diseases are unknown and the search for ultimate causes requires animal models. Animal models are important in understanding the progression of diseases in humans since it is possible to set up specific crosses in animals which is otherwise not possible in humans. Several animal models have been proposed based on whether the disease is induced or inherited. Completion of the chicken genome sequence has enhanced the chicken as the most important animal model for understanding human diseases. Despite these advantages, animal models are however not absolute solutions to elucidating human diseases as animals and humans have physiological differences as different species and have different protein functions. Furthermore there are differences in the immune systems between mammals and birds since birds have the bursa. However, identified genes may give a hint to the pathways of their involvement in disease progression.

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APPENDIX

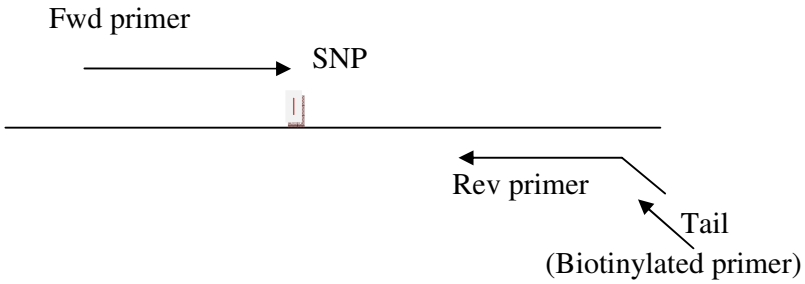

Fig 5. pyrosequencing assay for genotyping of SSc and vitiligo individuals.

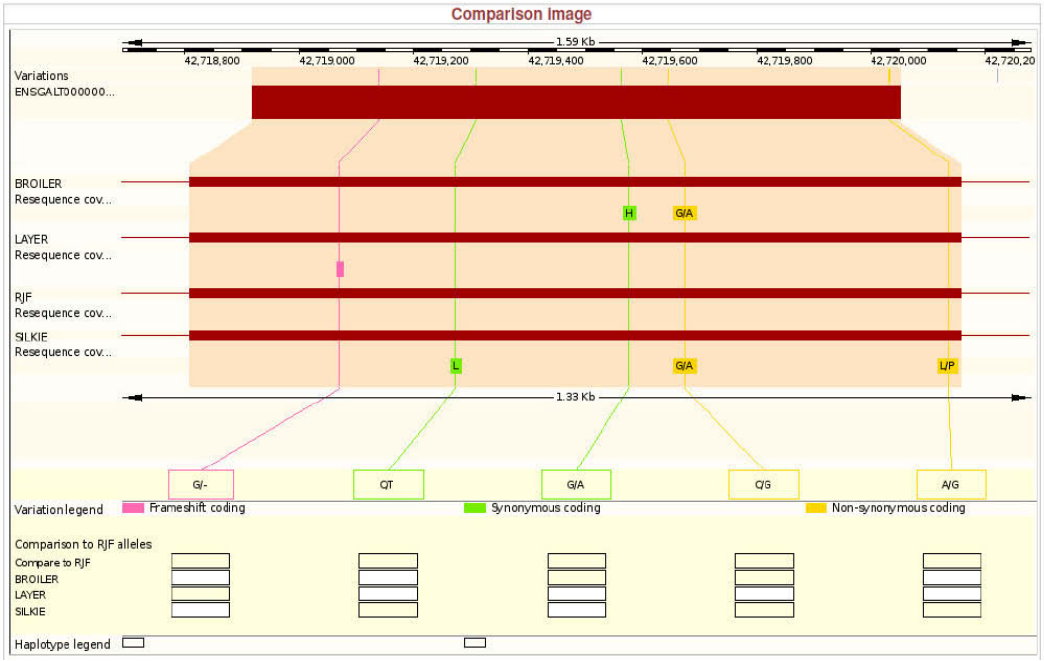

Fig 6. Comparison of the CCR8 SNPs in different chicken strains

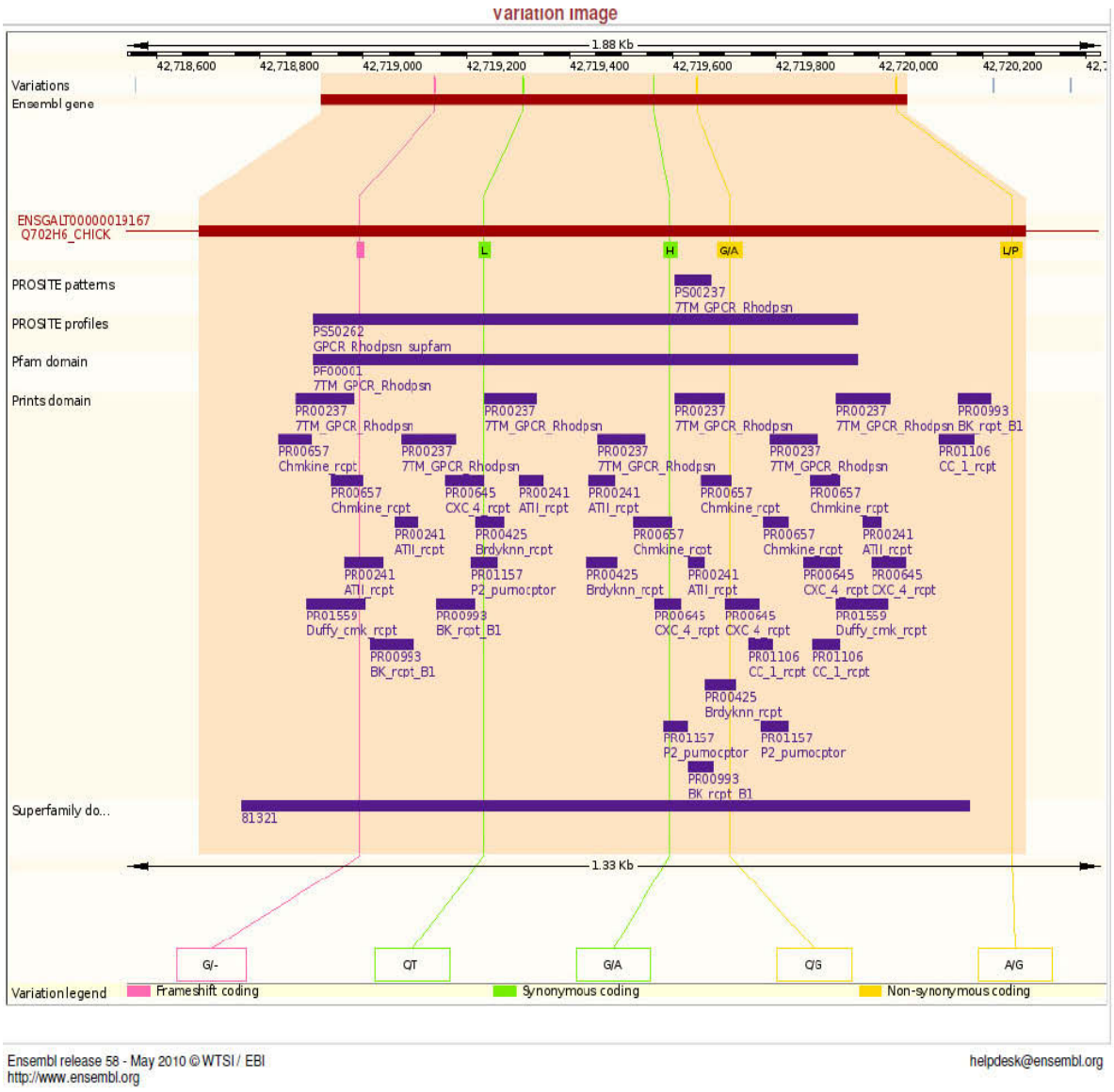

Fig 7. SNP variations in the CCR8 and proteins families coded for.

Table 7. Tissue samples collected form SSc and vitiligo chickens at slaughter.

Disorder		
Tissue	SSc	Vitiligo
Subcutaneous fat	√	√
Spleen	√	√
Testis	√	√
Breast muscle	√	√
Liver	√	√
Ovary	√	√
Heart muscle(apex)	√	√
Skin	√	√
Comb	√	X
Feather	√	√
Thyroid	√	√
Eyes	√	X

Table 8. Primers used for sequencing of the candidate genes in SSc.

Gene	**Primer**	**Primer sequence**
CCR8	CCR8_rev	TACCCATGACTCTTACCTCGTA
	CCR8_int1443_rev	GCATACACAGAGTGCACA
	CCR8_int2074_rev	CAAAAGAGGACTGTGAGGA
	CCR8_fwd	ATAACATCTGCTACGGAGAGGA
	CCR8_int2020_fwd	CACAGTGCCTTCAACAGA
CCL1	CCL1_fwd	GTGGTTTGTCGGTGGCAGCA
	CCL1_int1066_rev	CTGCTGGAAGATTGGGAA
	CCL1_int995_fwd	CACAGTACGCCTCTCTCA
	CCL1_rev	TGCATTGACCCTGCATTGAATCCA
SOCS1	SOCS1_fwd	CCGTAGCCACTGACGCTATGGA
	SOCS1_rev	TAAAAACCCAGGAAGCTGGA

Table 9. Primers used in genotyping of SNPs through pyrosequencing

chrom	Forwad primer	Reverse primer
3	CACGACGTTGTAAAACGACAACTTGCA TCAA TGCCAGTGA	ATGAGTCTCTCTTGTCRATCACA CA
9	CACGACGTTGTAAAACGACTTAGCAGC A TTGTTCTAGTTCCG	CAA AAAACCCCACATCTAGTTTC
21	CACGACGTTGTAAAACGACAGGCTG GGAGTTGGTGCACAG	GGGCTCAGGAAGCACGCAC
2	CACGACGTTGTAAAACGACCACTGAGT TTGT TGAATATGGCT	CATGTCACTATAACTTCACCTGC
12	CACGACGTTGTAAAACGACTGCTGTGA CTGG GTTGGAGG	CGCTGCCTGAGCAGAGCTGT
14	AACAGCAAGAGTAATATAAGAT	CACGACGTTGTAAAACGACTGA CTT AACACCCCATTTCTCTA
19	TCACACATGCAAACCAGACAC	CACGACGTTGTAAAACGACGGT ACAGCGCACATGGAAAAG

SNPs in QTL used in pyrosequencing

Chromosome 2 marker RS13673471

CAGATGCTAGTCAGGCTCACTGAGTTTGTTGAATATGGCTTGCATACAGTGATGGTTACTT
AAACACCTGCTCCTCCATTGCTTCCTGTGAATATTCAGT[C/T]GCAGGTGAAGTTATAGTG
ACATGCCAGGAGAAGGTAGCAGCTTAAACCAGTACAGTGTTTCAACATTTATTGATTTAT
AAGAAATCACAGAAGTGCACAT

Chromosome 12 marker RS14975805

GAAATGCTGTGACTGGGTTGGAGGCTCCCAGCAGCAGGCAGGAGGTCTGACACAGCGGG
GATGAACAAACTTCCGCTGGTACAAAAGGGATGACAGAACA[T/C]ACAGCTCTGCTCAGG
CAGCGGTGCCCATCAGAACCCCCTTGGGTyCATCCAGGCCCTTAGCAAAGCCACTCGGCA
GCCCTGAGCCyGGCGCACGGCCCCA

Chromosome 14 marker RBL1140

TGAAGGGTTCAAGAGTTAACAGTCAAAAATTGGCAGTGATCATTTTAATTTTTGATTTCTG
ACCAGCAGTATGCTGCAACAGCAAGAGTAATATAAGATC[A/G]TTGCTAGGCAAATCGTG
GCCCAAAAGAGCAGAGAAGGTAGAGAAATGGGGTGTTAAGTCACAGAGAACGATAACA
TCCAGGGCATACTCTGAAGAAGGTG

Chromosome 19 marker RS14123499

TGTGCCGTGACCTTAGCAAAAAGTGGTCGTCTCGGGAGCGCATGGAAATTCTCCCATTCT
AATCAAGAGGAATTAAACTTCACACATGCAAACCAGACAC[G/A]CCTCTTCTGAAAACCT
CAGTGCTAACAACGTGCGGTACATTAACTTGATTAATCCTAACGTAATCTTTTCCATGTGC
GCTGTACCGATGCCTTCTCCACC

R script

```
phen=as.matrix(read.table("phen.out"))
 batch=as.matrix(read.table("batch_sex.out"))

prob=as.matrix(read.table("KH_chr21.out",skip=2))

prob=subset(prob,phen[,2]!='-999')
batch=subset(batch,phen[,2]!='-999')
phen=subset(phen,phen[,2]!='-999')

 prob=prob[594:738,]
 batch=batch[594:738,]
 phen=phen[594:738,]


y=as.numeric(phen[,2])
sex=as.factor(batch[,2])
batch=as.matrix(batch[,3:6])
write(t(batch),ncol=4,file='temp_batch.txt')
batch=as.matrix(read.table("temp_batch.txt"))
ad=prob[,2]-prob[,3]
dom=prob[,4]+prob[,5]


lm1=lm(y~sex+batch+ad+dom)

glm1=glm(y~sex+batch+ad+dom,family=binomial)
```

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