GENOMICS-BASED ANALYSIS OF ANTIBODY RESPONSE TO SHEEP RED BLOOD CELLS IN CHICKENS

TUOYU GENG

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Animal and Poultry Sciences

Dr. Edward J. Smith, Chairman

Dr. Bennet Cassell

Dr. Eric M. Hallerman

Dr. Malcolm Potts

Dr. M.A. Saghai Maroof

Dr. Zhijian Tu

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Blacksburg, Virginia

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By

Tuoyu Geng

(ABSTRACT)

Immune response provides vertebrates an important mechanism to fight pathogens and to reduce the incidence of diseases. Defining the molecular basis of antibody response may facilitate genetic improvement in the immune response of animals to pathogens. For almost 4 decades, antibody titers in response to challenge by sheep red blood cells (anti-SRBC) have provided an investigative tool in the efforts to define molecular mechanisms that underlie vertebrate immune response. The overall objective of this dissertation research was to identify DNA markers associated with anti-SRBC response in chickens. Specific objectives were: to develop a resource population for QTL analysis for anti-SRBC, to identify DNA markers and genes associated with primary anti-SRBC, and to evaluate the allelic frequencies in non-selected chicken populations of candidate markers associated with either high or low anti-SRBC response. These objectives tested the hypothesis that genetic control of a chicken's response to SRBC is polygenic. The resource population developed consisted of F_1, backcross, and F_2 derived from reciprocal crosses of birds from parental lines in the 28^{th} generation of divergent selection for low (L) and high (H) anti-SRBC. The mean anti-SRBC titers of the parental lines were significantly different, with 11.5 for H and 2.6 for L ($P<0.05$). That for the 4 groups of F_2 progeny ranged from 6.3 to 7.5, while those of the 8 groups of backcross progeny ranged from 3.9 to 13.3. Four of 555 random primers used to screen the parental H and L anti-SRBC lines

were informative by amplifying seven line-specific fragments ($P<0.0025$). Each of the 7 line-specific fragments was converted to a sequence characterized amplified region (SCAR) within which single nucleotide polymorphisms (SNPs) were identified and tested for association with anti-SRBC. Only two of the seven SCARs in the parental lines were associated ($P<0.05$) with anti-SRBC level in the backcross resource population. Additionally, from analysis of the parental L and H anti-SRBC lines using microarrays, a total of 57 line-specific SNPs were also identified. Twenty of the line-specific SNPs were in and/or near genes previously reported to have immunity-related function. Microarray-based gene expression profiling of pooled RNA samples from L and H anti-SRBC birds identified three differentially expressed genes. In summary, this dissertation describes resources that include candidate SCARs and SNPs as well as differentially expressed genes that may be useful for the identification of genes that underlie antibody response.

Keywords: Antibody Response to Sheep Red Blood Cells, *Gallus gallus*, Quantitative Trait Locus

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DEDICATION

To my wife, Yan Jiang, and my daughter, Tianren Grace Geng

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ABBREVIATIONS

Ab	Antibody
AFLP	Amplified fragment length polymorphism
ALV	Avian leukosis virus
AMI	Antibody-mediated immunity
ANOVA	Analysis of variance
Anti-SRBC	Antibody response to sheep red blood cell
APC	Antigen presenting cell
BC	Backcross
BLAST	Basic local alignment search tool
CD	cluster designation
cDNA	Complementary DNA
cM	centiMorgan
CMI	Cell-mediated immunity
DC	Dendritic cell
DE	Differentially expressed
EST	Expressed sequence tag
FDR	False discovery rate
GLM	General linear model
GO	Gene ontology
H	High antibody response to sheep red blood cell
IBD	Identical by descent
Ig	Immunoglobulin
IL	Interleukin
L	Low antibody response to sheep red blood cell
LD	linkage disequilibrium
LOD	Logarithm of odds
Mb	Mega-base
mDC	Matured dendritic cell
MDV	Marek's disease virus

MHC	Major histocompatibility complex
mRNA	Message RNA
NIL	Near-isogenic line
PCR	Polymerase chain reaction
PD	Protein Domain
PRR	Pattern recognition receptor
QTL	Quantitative trait locus
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragement length polymorphism
Rfp	Restriction fragement pattern
RH	Radiation hybridization
RIL	Recombinant inbred line
RIR	Rhode Island Red chicken
RT-PCR	Reverse transcription-polymerase chain reaction
SCAR	Sequence characterized amplified region
SNP	Single nucleotide polymorphism
SRBC	Sheep red blood cell
SSR	Simple sequence repeat
STS	Sequence-tagged site
T_c	Cytotoxic T cell
TDT	Transmission disequilibrium test
T_h	Helper T cell
TLR	Toll-like receptor
VDJ	Variable-Diversity-Joining
VNTR	Variable number of tandem repeats
WPR	White Plymouth Rock chicken

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CHAPTER 1

Introduction

Focus on agricultural and biomedical studies has shifted from analysis of monogenic to polygenic traits. Although new analytical tools and genomic data have emerged in the post-Human Genome Project era, identification of genes underlying polygenic traits remains a significant challenge. This is probably because identification and characterization of most genes with small effects are complicated by multiple influences of genetic and environmental noise, epistasis, and epigenetics (Andersson and Georges, 2004).

An economically important trait in livestock and poultry for which gene identification continues to be a challenge is immune response, which can be either cellular or humoral. The primary effectors of cellular immunity are various immune cells, including neutrophils and cytotoxic T cells (Faurschou and Borregaard, 2003; Bleackley, 2005; Waterhouse et al., 2004), while those for humoral immunity are antibodies (Harnett et al., 2005). This dissertation research focused on humoral or antibody response, a polygenic trait (Feingold et al., 1976; Biozzi et al., 1979; Gross and Siegel, 1980).

Antibodies provide vertebrates an important mechanism to resist invasions and infections from diverse pathogens. Therefore, a strong antibody-producing ability could have important implications in animal agriculture and human medicine. Antibody response includes different cellular and molecular components, as well as cross-communication with cell-mediated immunity, suggesting that the underlying mechanism is complex. Efforts have focused on defining the molecular basis of this mechanism in different animals, including chickens (Berek, 1989; Brunner et al., 1995; George et al., 1992; Becker et al., 2005). Identification of the

polygenes or quantitative trait loci (QTL) associated with antibody response to SRBC, as proposed here, will provide a foundation to significantly advance these efforts.

Antibody levels are important dynamic parameters of immune response as they partially reflect the potential of an animal to resist pathogen infection. The evidence that antibody level is controlled by multiple genes, thus leading it to the possibility of improvement by selection, has been based on work in diverse species including mouse (Feingold et al., 1976; Biozzi et al., 1979; Ibanez et al., 1988), chicken (Siegel and Gross, 1980; Van der Zijpp et al., 1983), and guinea pig (Ibanez et al., 1980). In addition to many reports on the genetics of immune response, some genomic studies have described candidate DNA markers associated with antibody production in lines divergently selected for high or low levels of antibodies produced in response to a challenge by heterogeneous erythrocytes such as sheep red blood cells (Puel et al., 1995, 1996).

Compared to specific antigens such as a virus or bacterium, use of heterogeneous erythrocytes in antibody response-related studies has two distinct advantages. First, heterogeneous erythrocytes are multi-determinant and strongly immunogenic antigens. This heterogeneity increases the likelihood of identifying more QTLs associated with antibody production. Studies in mice involving heterogeneous erythrocytes described by Puel et al. (1995, 1996), for example, reported genes including background genes unlinked to immunomodulatory genes at the major histocompatibility complex (MHC) and the immunoglobulin heavy chain (*Igh*) loci that appeared to influence antibody response. Second, selection for antibody response to heterogeneous erythrocytes may improve general natural disease resistance of vertebrates. This is supported by the observation that selection for antibody response to heterogeneous erythrocytes was correlated with disease resistance to multiple pathogens (Gross et al., 1980).

Genetic lines divergently selected for high or low antibody titers to SRBC (anti-SRBC) have been established in both mouse (Biozzi et al., 1972; Feingold et al., 1976), chicken (Siegel and Gross, 1980; Van der Zijpp et al., 1983), and guinea pig (Ibanez et al., 1980). These lines provide unique resources for dissecting the molecular basis of antibody response. This is because selection leads to fixation or near fixation of a favorable allele and the flanking regions in the selected populations or lines (Andersson and Georges, 2004; Siegel et al., 2006). This change in allelic frequency due to selection facilitates the identification of QTLs associated with the selected trait through linkage analysis or linkage disequilibrium (LD)-analysis. For example, from screening mice divergently selected for high or low anti-SRBC titers, Puel et al. (1995, 1996) described several chromosomal regions containing QTL with significant effect on anti-SRBC as well as genes located in MHC and Igh loci. However, linkage analysis only narrows the location of a QTL to a region containing numerous genes, but rarely leads to the identification of the causative genes for the trait.

The limitations of linkage analysis for QTL identification are believed to be overcome by LD, as it has been shown to facilitate the identification of QTLs with small effects as well as a higher statistical power (Risch and Merikangas, 1996). Based on frequency differences, genes that affect a trait of interest may be identified through LD-based QTL mapping (Keightley et al., 1993). Genome-wide scanning tools also have been used in LD-based identification of QTLs or genetic markers associated with QTLs. Although the LD-based approaches are advantageous in mapping efficiency, their use in QTL mapping in livestock and poultry remains limited.

A tool that has been used to search for markers associated with QTLs in genome-wide scans is the randomly amplified polymorphic DNA (RAPD) technique. First described by Williams et al. (1990), RAPD involves the use of arbitrary primers to amplify DNA fragments

when they bind to a DNA template. Using RAPD in a genome-wide scan, Martin et al. (1991) and Gu et al. (1998) identified markers linked to a *Pseudomonas* resistance gene in tomato and to progressive rod-cone degeneration in dogs, respectively.

Microarray-based expression profiling is another genome-wide analysis tool that has recently been used for QTL analysis. It enables parallel comparisons of gene expression levels on a genome scale. Combining microarray analysis with QTL analysis may facilitate the identification of causative genes from candidate markers for the selected phenotype. For example, Buhr et al. (2006) identified three major candidate genes for experimental inflammatory bowel in human using this approach. To our knowledge, microarray-based transcription profiling for antibody response to SRBC in vertebrates has not been described. Such a study may facilitate identification of genes influencing anti-SRBC titers by complementing linkage- and LD-based QTL analysis.

To date, anti-SRBC candidate genes reported from previous investigations have not provided markers with a universal appeal or that are informative in other chicken populations (Yonash et al., 2001; Siwek et al., 2003; Zhou et al., 2003). Therefore, this dissertation research had, as its primary objective, the identification of candidate markers associated with the level of anti-SRBC titers in the chicken. To accomplish this objective, an appropriate resource population was necessary. The first part of the dissertation describes this resource and its use to discover candidate markers for anti-SRBC. In the second part, the genome distribution of the candidate markers was determined using genetic and radiation hybridization mapping approaches. Further, the markers were evaluated in backcross population and PCR-RFLP methods were developed for genotyping 30 birds from each of 7 other chicken populations with one of the informative SNPs within the markers. In the final part of the dissertation, randomly bred birds were evaluated

through microarray-based gene expression QTL analysis for association of levels of anti-SRBC titers with candidate markers identified in the resource population. The dissertation research is primarily a resource-driven project, although it is based on the hypothesis that differences among chickens for anti-SRBC titers are polygenic. The dissertation will describe the resources that could provide a foundation for improving the natural immune capacity of chicken and thus reduce the use of antibiotics and the susceptibility to unknown pathogens.

CHAPTER 2

Review of literature

2.1 Introduction

The chicken (Gallus gallus) is important both to agriculture as a food animal and to biomedical science as a model for biomedical research, respectively. After beef cattle and hog, the chicken is the third largest food animal in consumption and gross farm receipts. As a model organism, the chicken has provided important insights into vertebrate biology that includes genetics and developmental biology (Osterrieder et al., 2006; Dupin et al., 1998; Mello and Tuan, 1998; Pain et al., 1996). For example, the chicken has been used as a model for investigating the heredity of spontaneous autoimmune thyroiditis (Cole, 1966; Cole *et al.*, 1968), genetic dwarfism (Hull and Harvey, 1999), and vertebrate limb development (Tickle, 2004). The chicken's use as a model organism is due to several advantages including a short generation interval, large family size, and extensive published knowledge about its physiology, nutritional requirements, and genetics. Discoveries about the chicken's immune response, the primary goal of this dissertation, may prove applicable to other vertebrates.

The immune system is of primary importance in vertebrates, including the chicken, because of its significant impact on general well-being and productivity. It is a critical part of the defense mechanism of vertebrates against pathogens. Immune response is the function of the immune system, which is either innate or adaptive. The innate immune system is characterized by quick response with low level of specificity for antigen recognition. Inflammation is one of the mechanisms used by the innate immune system to control the spread of pathogens. It mainly relies on the functions of neutrophils and mononuclear phagocytes. In contrast, the adaptive immune system has a mechanism to mount stronger and more efficient immune response to the

invading pathogens. This is because the B and T lymphocytes, which form the cellular components of immune response, have the ability to recognize specific antigens. B cells are primarily responsible for antibody-mediated immunity, although one important mechanism by which they are activated requires the function of helper T cells (Holdsworth et al., 1999; Infante-Duarte and Kamradt, 1999; Mitchison et al., 1999; Berenson et al., 2004). In comparison, T cells are the major effectors of cell-mediated immunity. Although the innate and adaptive immune systems have separate mechanisms and distinctive features for fighting pathogens, they are interconnected by dendritic cells, the professional antigen presenting cells, and/or by the complement system. Both systems contribute to natural disease resistance of vertebrates. Several genetic factors influencing both antibody response and disease resistance have been identified, including the major histocompatibility complex, immunoglobulin, cytokines and endogenous viral genes.

Divergently selected lines that originate from a non-inbred base population provide unique resources for studying the molecular basis of quantitative traits like immune response. Compared to inbred lines, the commonly-used resource populations, the divergently selected lines provide some advantages for QTL mapping. One advantage is that QTLs associated with the selected trait could be directly identified based on the difference in allelic frequency of markers between the divergently selected lines. This is because selection could lead to fixation or near-fixation of a favorable allele and an unfavorable and its flanking regions in each of the respective lines (Andersson and Georges, 2004). If appropriate genome-wide scan tools, such as RAPD, are employed to screen for line-specific or nearly line-specific markers, the QTLs under strong selection could be identified without using linkage analyses. Another advantage is that most QTLs that underlie a selected trait could be identified because the divergently selected lines

that originate from a non-inbred base population generally do not have the problems associated with inbreeding. For example, it is possible for a QTL to be non-polymorphic or marginally different in gene effect between inbred lines because inbreeding greatly reduces polymorphism.

Experimental lines of both mice and chickens divergently selected for high or low anti-SRBC response have been developed. The mouse lines have been used for identification of QTL or DNA markers associated with anti-SRBC. However, this study was based on linkage analysis. One of the shortcomings of linkage-based QTL analysis is that it cannot provide information about the precise location of a QTL. Linkage disequilibrium-based QTL mapping, which utilizes LD resulted from historical recombination events to map QTL, may be better in QTL mapping efficiency. For example, it can be used for identifying QTLs with small effects, QTL fine-mapping, and increasing the power of QTL detection given a small sample size (Risch and Merikangas, 1996).

In addition to structural genomics approaches, functional and *in-silico* genomics also have been used for QTL analysis. In the functional genomics, microarray-based transcription profiling provides a powerful tool for QTL identification. However, this technology is still developing. Issues of concern that relate to variability and data analysis should be considered carefully in microarray experiments focused on identifying QTLs. In comparison with the experimental approaches mentioned above, the *in silico* genomics-based analysis provides a convenient approach for transferring QTL information from one species to another.

In this review, the roles of the chicken as a food animal and a model animal for immune-related QTL analysis are discribed. Emphasis will be on recent findings from genomics investigations that appear to increase our understanding of the molecular mechanisms that underlie vertebrate immune response, especially the regulation of antibody levels. The review

includes a discussion of the value of resource populations to mapping of QTLs associated with economically important traits in livestock and poultry. This will be followed by a review of genomics-based approaches for QTL analysis. The limits of the new and old QTL analysis methods will be discussed. Specific reports from published QTL analysis investigations of anti-SRBC response will be summarized.

2.2 The domestic chicken

2.2.1 The chicken as a food animal

The chicken (*Gallus gallus*) is raised both for meat and egg, making it the most significant avian food species. Several characteristics of the chicken contribute to its importance as the foremost poultry and egg bird. First, chicken meat and eggs provide one of the most important sources of animal protein for the human population worldwide. In 2005, chicken meat accounted for about 41% of meat produced in the United States with per capita consumption of approximately 45 kilograms of chicken meat. During that period, about 14 kilograms of eggs per capita were consumed (United States Department of Agriculture, 2006). Similar growth in chicken meat and egg consumption was observed in developing countries. In China, for example, a significant fraction of meat consumed in 2005 was poultry, at 19%. The per capita consumption in 2005 was about 10 kilograms of poultry meat and 21 kilograms of eggs (Ministry of Agriculture, PRC, 2005).

Second, the market share for chicken meat and eggs continues to grow. Broiler production in the U.S. grew by 33% in the last decade to about 35 billion pounds in 2005 (Department of Agriculture, US, 2006), while the growth in China was similar at about 29% (Ministry of Agriculture, PRC, 2005). This growth is expected to continue. According to Global

Food Outlook 2020 (Rosegrant et al., 2001), increasing demand for high quality protein in the developing world is expected to be one of the most important trends in the future of agriculture with chicken meat expected to be at the forefront of the demand.

A third advantage of the chicken is that establishing a functional production operation is usually more cost-effective than for other major meat animals. Finally, cattle and swine, the other major meat animal species with which chicken competes for consumer demand, are not compatible with certain religious practices, whereas chicken consumption appears to be nearly universal.

2.2.2 The chicken as a model for vertebrate immune response

The chicken has been used a s a unique model organism for studying immune response due to the following immune-related features. First, the chicken has bursa of Fabricius, the site of maturation of B lymphocytes first described by Cooper et al. (1966). It is a distinct immune organ, which provides an easy opportunity to study B cells and B cell-related antibody response. Second, the generation of immunoglobulin diversity in the chicken provides an example illustrating that the regulation of immune response at the DNA level is different between birds and mammals. In the chicken, immunoglobulin diversity mainly results from somatic mutation (probably via gene conversion using sequences from flanking pseudogenes) other than the variable-diversity-joining (VDJ) recombination mechanisms that predominate in mammals (Reynaud et al., 1994). Last, as critical component of immune response, the major histocompatibility complex, represents in the chicken a “minimal essential MHC” because it only spans 92 kilobases, while its counterpart in the human, denoted HLA for human leukocyte antigen, spans 3.6 megabases.

2.3 The immune system

The immune system is a set of mechanisms that protects an organism from invasion or infection by identifying and killing foreign or foreign-like substances, including pathogens such as bacteria, viruses and fungi, as well as offer protection from parasites (Accolla, 2006). Immune response is broadly defined as the defense of the host as a result of its immune system to pathogens and parasites. Because pathogens are diverse and continue to evolve new ways to avoid detection by the immune system, it is extremely difficult for host organisms to defend themselves from invasions and infections by pathogens (Essex, 1997; Fleck, 2004). A sophisticated and evolving mechanism is required for performing this task successfully. Vertebrates have developed such a mechanism-the immune system. The system can be either innate or adaptive. Innate and adaptive immune systems are interconnected and function in different ways. Improving innate and adaptive immune response simultaneously or separately may increase a species' natural disease resistance (Pitcovski et al., 2001; Pevzner et al. 1981; Bajaj-Elliott and Turner, 2005; Yuan and Walker, 2004).

2.3.1 The innate immune system

The innate immune system is an evolutionarily ancient form of host defense against infection, providing a quick response with low antigen-specificity. It employs multiple mechanisms to recognize and control the spread of pathogens, including humoral and chemical barriers such as inflammation, and cellular barriers (Hackett, 2003). The humoral and chemical barriers involve many humoral factors such as mucosal secretions and serum factors including complement, certain cytokines and natural immunoglobulins. The cellular barriers rely on the functions of the cells, including natural killer cells, macrophages, dendritic cells (DCs) and granulocytes (Hackett, 2003).

Innate immune defense is quickly activated when pathogens break through host surface barriers. The latter includes mechanical (e.g., skin), chemical (e.g., lysozyme, phospholipase A, defensins), and biological (commensal flora, pH) barriers. When exogenous immune-related mediators are released from locally damaged tissue (Gallucci and Matzinger, 2001), they interact with histiocytes (macrophages and neutrophils), dendritic cells, and mast cells at the site of injury. The interactions subsequently result in the production of endogenous mediators. The immune defense system recruitment is then promoted by the endogenous mediators through inflammation, which is characterized by locally increased endothelium permeabilization, concomitant with upregulation of integrin and other adhesion molecule expression on the endothelium. Histiocytes and other leukocytes are subsequently recruited from the blood to the endothelium by chemokines released from the endothelium (Mackay, 2001; Murdoch and Finn, 2000). Among the recruited leukocytes, neutrophils and monocytes are typically the dominant migratory leukocytes in the innate defense responses (Murdoch and Finn, 2000).

Neutrophils (or polymorphonuclear neutrophilic granulocytes) and mononuclear phagocytes (monocytes and macrophages) are central to the acute inflammatory reaction (Kobayashi et al., 2005), and have a wide distribution throughout the body tissues and organs. Neutrophils are the most abundant type of phagocyte, normally representing 50% to 60% of the total circulating leukocytes. They are key effector cells of the innate immune system (Faurschou and Borregaard, 2003). When they migrate to sites of infection, they are activated and a cascade of defense mechanisms is initiated. Neutrophils recognize and engulf pathogens, and subsequently kill and degrade pathogens via the production and finely tuned release of reactive oxygen species (superoxide, hydrogen peroxide, and hypochlorous acid) and antimicrobial and proteolytic granule proteins, which are delivered to the phagosomes and to the extracellular

environment (Segal, 2005; Faurschou and Norregaard, 2003). In addition, activated neutrophils synthesize chemokines and cytokines, which recruit and regulate the inflammatory responses of other effector cells including macrophages, T cells, and neutrophils themselves (Theilgaard-Monch et al, 2004). Finally, activated neutrophils initiate an apoptotic program, which facilitates resolution of inflammation and prevents tissue damage caused by necrotic cell lysis, and spilling of cytotoxic effector proteins and reactive oxygen species to the extracellular environment (Kobayashi et al., 2003a, b).

The mononuclear phagocytes (macrophages and monocytes), including DCs, are also important to the innate defense response. One important role of the macrophage is the removal of necrotic debris from the site of infection. Macrophages also present antigenic molecules of pathogens to a helper T cell and play a role in cell-mediated immunity. This presentation is done after pathogens are engulfed and digested by macrophages. In this process, antigenic peptides generated from the digested pathogens are integrated into the cell membrane and displayed by attaching them to MHC class II molecules (Brode and Macary, 2004). The role of macrophages as antigen presenting cells (APCs) sets up a link between the innate and adaptive immune system. Although macrophages had been considered to be APCs, the principal APCs are the DCs (Steinman, 1991). In contrast to other cells that can function as APCs (e.g., macrophages and B cells), the main function of DCs is antigen presentation (Banchereau et al., 2000; Steinman, 1991). Dendritic cells are the major link between the innate and adaptive immune systems, as they present antigen to T cells, one of the key cell types of the adaptive immune system.

Together, a variety of cellular and humoral components with different functions endow the innate immune system with power to fight invading pathogens. However, the ability to

distinguish pathogens from self is a prerequisite for immune system to function beneficially to the host.

The innate immune system distinguishes pathogens from self with pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (Hoffmann and Reichhart, 2002; Janeway and Medzhitov, 2002). The latter is the structural motifs shared by related species of microbes. These motifs include cell walls, components of microbial membranes, DNA and proteins. PRRs are highly conserved and exist before the evolutionary divergence of the plant and animal kingdoms (Hoffmann and Reichhart, 2002). Therefore, they only recognize a fixed structural motif and do not undergo rearrangement.

Toll-like receptors (TLRs) are one important category of PRRs (Mukhopadhyay et al., 2004). They mediate ligand-binding specificity. Intracellular signaling pathways are initiated by their bindings to the corresponding ligands. As the consequences of TLR activation, antigen presentation, phagocytosis and apoptosis (cell death) are induced. TLRs also induce expression of a number of inflammatory cytokines and chemokines and rapidly alter cell trafficking and recruitment. In addition, TLR activation promotes DC maturation by upregulation of MHC proteins and costimulatory molecules, thus providing the two necessary signals for T cell activation. Other PRRs include mannan-binding lectin, C-reactive protein, serum amyloid protein, macrophage mannose receptor, and macrophage scavenger receptor (Mukhopadhyay et al., 2004).

2.3.2 The adaptive immune system

When pathogens break through the innate immune system, the adaptive immune system will be activated and mount stronger and more efficient attacks on the invading pathogens. Unlike innate immunity, adaptive immunity is a slow response with high antigen-specificity. It

requires the recognition of specific antigens during antigen presentation. Antigen specificity allows for the generation of responses that are tailored to specific pathogens or pathogen-infected cells. The capacity for adaptive immunity relies on its highly specialized systemic cells and processes that eliminate pathogenic challenges.

Lymphocytes including B cells and T cells are the effector cells of the adaptive immune system. Both types of cells are derived from the same pluripotential stem cells, and they are indistinguishable from one another until after they are activated. B cells play roles in the humoral immunity (or antibody-mediated immunity, AMI), whereas T cells primarily involve cell-mediated immunity (CMI) (Harnett et al., 2005).

2.3.2.1 Antibody-mediated immunity

The principal function of B cells is to make antibodies (Abs) against soluble antigens. B cells are activated and differentiated into plasma B cells, which in turn secret Abs as the major effector molecules of antibody-mediated immunity, when pathogens evade from innate immune response. However, this differentiation requires not only antigen recognition, but also T cell dependent or independent activation (Balazs et al., 2002; Bernard et al., 2005). Each B cell has a unique receptor protein (B cell receptor) on its surface that will bind to one particular antigen. The B cell receptor is a membrane-bound immunoglobulin, which involvs B cell activation. Once a B cell encounters its cognate antigen and receives an additional signal from a helper T cell, B cell will be activated and differentiated into Ab-producing cell (McHeyzer-Williams, 2003).

The activated B cells also differentiated into memory B cells, which are long-lived cells. Memory B cells provide the adaptive immune system with the ability to mount a quick Ab response specific to the pathogen presented in primary infection if the same pathogen is

encountered. This ability is attributable to their specific recognition of the antigen and to their quick proliferation, which leads to a new generation of Ab-producing plasma cells.

The mechanisms by which B cells are activated can be classified into T cell-independent and T cell-dependent activation. During T-cell dependent activation, a processed antigen is presented by an APC presents to a helper T (T_h) cell, which is subsequently primed by the presentation (Konig et al., 2002; McHeyzer-Williams et al., 2006). If a B cell processes and presents the same antigen with a class II MHC protein to the primed T_h cell, the T cell releases cytokines that activate the B cell (Janeway et al., 2001). Although most antigens are T-cell dependent, B cells are also activated through PRRs in the absence of T cell help. During the T cell-independent activation, antigen is presented in a way that causes cross-linking of antibodies on the surface of B cells. B cell activation then may either proceed directly or undergo an intermediate differentiation step.

2.3.2.2 Cell-mediated immunity (CMI)

T cells play a central role in CMI, which does not involve antibodies (Erf, 2004; Bleackley, 2005; Walter and Santamaria, 2005; Waterhouse et al., 2004). T cells travel to and develop in the thymus. They can be distinguished by the presence of T cell receptor on their cell surface. T cells are divided into several subsets, including cytotoxic T cells (T_c cells), helper T cells (T_h cells), memory T cells, regulatory T cells, natural killer T cells and γδ T cells (Williams and Bevan, 2007; Street and Mosmann, 1991). Among these subsets, T_c cells are the major type of T cells responsible for antigen-specific CMI (Moser and Eberl, 2007).

Cytotoxic T cells derive from double-positive T cells that have both the CD4 (cluster designation 4) and CD8 co-receptors (Oehen and Brduscha-Riem, 1998; Blanco et al., 2005). The double-positive T cells (CD4+CD8+) first are exposed to a wide variety of self-antigens in

the thymus. The strength of their binding to MHC-presented self-antigens determines if these cells will survive or not (Kaufman et al., 1992). If it is too strong, the cells will undergo apoptosis because their propensity to become autoreactive, which could lead to autoimmunity. This process is called negative selection. If the binding is too weak, the cells will undergo positive selection, in which apoptosis is initiated because it is difficult for them to recognize MHC-protein complexes. Those cells that survive positive and negative selections differentiate into single-positive T cells (either CD4+ or CD8+). This differentiation is dependent on whether their receptors recognize an MHC class I-presented antigen (CD8) or an MHC class II-presented antigen (CD4). Only the CD8+ T cells that will mature into cytotoxic T cells after their receptors bind to a class I restricted antigen (Oehen and Brduscha-Riem, 1998).

The activation of T_c cells is dependent on several simultaneous interactions between molecules expressed on the surface of the T cell and molecules on the surface of the APC. An interaction is between the T_C cell receptor and a peptide-bound MHC class I molecule on the antigen presenting cell. Another interaction is between the CD8 coreceptor and the class I MHC molecule (Rock and Shen, 2005), which may stablize the interaction between the T_C cell receptor and a peptide-bound MHC class I molecule. The two interactions form the first signal for the T cell activation. The interaction between the CD28 molecule on the T cell and either CD80 or CD86 on the surface of the antigen presenting cell forms the second signal. CD80 and CD86 are costimulators of T cell activation. The second signal can be assisted by stimulating the T_c cell with cytokines released from helper T cells. The activated T_c cells undergo clonal expansion with the help of interleukin-2, which is a growth and differentiation factor for T cells. Once T_c cells are exposed to infected/dysfunctional somatic cells, they release cytotoxins that form pores in the target cell's plasma membrane and cause ions and water to flow into the target cell. This

eventually leads to the expansion and lysis of the target cell. T_c cells also release granzyme to target and destroy infected somatic cells because granzyme can enter the target cells via the perforin-formed pore and induce apoptosis by activation of caspases (Ashton-Rickardt, 2005).

2.3.3 Innate and adaptive immunity

2.3.3.1 Links formed by dendritic cells

As mentioned above, dendritic cells, as professional APCs, are the major link between innate and adaptive immunity (Groothuis and Neefjes, 2005; Heath et al., 2004; Brode and Macary, 2004). This link is established by multiple interactions of DCs with T_h cells, T_c cells and B cells in either an MHC- dependent or independent manner (Corthay, 2006).

Dendritic cells can internalize antigen, or cells such as tumor, virus-infected, and apoptotic cells (Banchereau et al., 2000). The internalized material is degraded into antigenic peptides within the endosomal system. There are two options to process the antigenic peptides, which involves either MHC class II or MHC class I molecules (Guermonprez et al., 2002; Steinman et al., 1999; Thery and Amigorena, 2001). In the former case, the antigenic peptide-containing endosomal structures fuse with specialized structures that contain MHC class II molecules. The antigenic peptides then interact with the MHC class II molecules, which is capable of recognizing the peptide amino acid sequence. These peptide-loaded MHC class II molecules are subsequently transferred to the cell surface, where they are stably expressed - an important characteristic of a mature DC (mDC; Pieters, 2000). When the muature dendritic cells encounter naive CD4+ T cells, the first signaling pathway will be activated by the interaction between the T cell receptors and the peptide-bound MHC class II molecules. As a result of this activation, the naive CD4+ T cells will mature into T_h cells (Malnati et al., 1992). The second signaling pathway that is also necessary for maturation of the CD4+ T cells into T_h cells is

activated by the interaction between CD28 on the $CD4^+$ T cell and the proteins CD80 or CD86 on the professional APCs. The CD4+ T cells can differentiate into Type 1 (T_h1) and Type 2 (T_h2) helper T cells in the process of maturation (Holdsworth et al., 1999; Infante-Duarte and Kamradt, 1999; Mitchison et al., 1999; Berenson et al., 2004). This differentiation is based on the specific cytokines they produce. T_h1 cells produce interferon-gamma and tumor necrosis factor beta, which induce the proliferation of T_c cells. In comparison, T_h2 cells produce interleukin- 4, 5 and 13, which induce B cell Ab class switching and increase Ab production by stimulating B cells into proliferation (Becker et al., 2005). T_h2 cells, therefore, are considered necessary for the full maturation of the humoral immune system.

If the internalized material is transferred to the cytosol for processing, the latter case, the antigenic peptides will bind to MHC class I molecules of DCs and be presented on the cell surface to T_c lymphocytes (Brode and Macary, 2004; Heath et al., 2004; Thery and Amigorena, 2001). This process is critically important in defending against intracellular pathogens such as viruses and certain bacteria. The antigen-bound MHC class I molecules then interact with the T cell receptors on the naïve CD8+ T cells in a similar way as the antigen-bound MHC class II molecules do (Melief, 2003). This interaction stimulates the naïve T_c cells to mature into effector cells. Similarly, the additional involvement of costimulatory receptors is required to stablize the interaction between the DCs and the T_c lymphocytes (Sproul et al., 2000).

DCs also interact with B cells in a MHC-independent manner. DCs can directly cluster with B cells *in vivo* (Kushnir et al., 1998) and take part in B cell activation through antigen delivery (Litinskiy et al., 2002; Ludewig et al., 2000). The DC-B cell interaction leads to the differentiation of B cell into Ab-producing cells in a T cell-independent style (Balazs et al., 2002;

MacPherson et al., 1999). This interaction does not need a processed antigen and the involvement of MHC.

2.3.3.2 Links formed by the complement system

The serum complement (C) system is a major component of innate immunity. It not only participates in inflammation, but also enhances adaptive immunity by releasing cleavage products that interact with surface receptors on both B and T cells (Reid and Porter, 1981; Fearon and Carroll, 2000). These receptors include specific Ab and innate recognition proteins such as TLRs (Janeway and Medzhitov, 1999; Barton and Medzhitov, 2002), manna-binding lectin (Epstein et al. 1996), ficolins (Matsushita and Fujita, 2002), C-reactive protein (Szalai et al., 1997), C1q (Korb and Ahearn, 1997; Taylor et al., 2000) and natural immunoglobulin M (IgM; Boes et al., 1998; Baumgarth et al. 2000; Reid et al., 1997; Toapanta and Ross, 2006).

The early reports of binding of complement 3 to circulating lymphocytes (Dukor et al., 1971) and follicular dendritic cells within lymphoid follicles (Papamichail et al., 1975) indicated the involvement of the complement system in adaptive immunity. Complement 3 is the central component shared by the three different activation pathways of the complement system, including classical, lectin and alternative. Complement enhances B cell immunity primarily via complement receptors CD21 and CD35, both of which binds the complement 3 products including iC3b, C3d,g and C3d (Molina et al. 1994). The two receptors are both expressed mainly on B cells and follicular dendritic cells. On B cells, CD21, CD19 and CD81 can form a receptor complex that is able to co-engage with B cell antigen receptor (Matsumoto et al., 1991). This coengagement can lower the threshold of B cell activation, and provides an important survival signal for the cognate B cells when the C3d-coated antigen is uptaken (Carter and Fearon, 1992). In addition, complement enhances B cell immunity by localizing antigen to

follicular dendritic cells within lymphoid follicles (Fang et al. 1998; Kemper and Atkinson, 2007; Sahu and Lambris, 2001).

The complement system also may play a role in T cell immunity to a specific antigen. Its role reflects on the ability to mark the antigen as foreign and to be recognized by specific receptors. In addition, the chemoattractant receptors C3aR and C5aR, which are the byproducts of complement activation, may alter T cell responses. However, early studies that examined the function of complement in T cell priming suggested that the involvement of complement in B cell immunity was more important than that in T cell immunity (Isaak et al., 2006; Korthauer et al., 1993; Krummel and Macara, 2006).

2.3.4 Characteristics and dynamics of antibody-mediated immunity

Specific immune responses are the major type of AMI, in which antibodies produced by plasma cells attach to invading microorganisms and mark them for destruction or prevent them from infecting cells (Butler, 1998; Doria and Frasca; 1997). Their general characteristics include self/non-self discrimination, memory, and specificity. Specific Ab responses can be divided into primary Ab response and secondary Ab response. Primary response refers to the first time a host is exposed to the antigen. Antibodies made upon first exposure to an antigen are mostly of the class IgM. Secondary response refers to subsequent exposure. The class IgG antibodies are dominant in the secondary response. Therefore, immunoglobulin antibodies undergo a process called Ab class switch from primary to secondary Ab response. In addition, affinity maturation occurs during the secondary response, in which the IgG Ab increases its affinity to the antigen progressively, especially after low doses of antigen. This phenomenon could be explained by either clonal selection or somatic mutations in which the antibodies are fine-tuned to be of higher affinity after Ab class switch (Janeway et al., 2004).

The primary Ab response also is different from the secondary Ab responses in kinetics. In the primary response, there is an initial lag phase when no Ab can be detected after the first challenge with antigen. In this phase, the antigen is recognized as foreign, and the cells start proliferation and differentiation in response to the antigen. The duration of this phase varies with the antigen used, but it is usually 5-7 days (Biozzi et al. 1972; van der zijpp et al. 1983b). The lag phase is followed by exponential phase, in which the Ab titer rises logarithmically to a plateau. In the log phase, the B cells differentiate into Ab-producing plasma cells. The plateau phase subsequently is reached as Ab synthesis is balanced by Ab decay so that there in no net increase in Ab concentration. During this phase, the antibodies bind to the antigen or are naturally decomposed, and subsequently are cleared from circulation. The last phase of the primary Ab response is called the decline phase, in which the degradation of Ab exceeds its synthesis. As a result, the level of Ab may reach baseline levels. In contrast, the lag phase is shorter and the plateau and decline phases are extended in the secondary response. In the plateau phase, the secondary response is more rapid than the primary response, and the plateau of Ab titer is typically 10 times higher. Unlike the primary response, the decline phase in the secondary response is not quick and Ab may persist for long time, even a lifetime.

Evidence has shown that the dynamics of Ab response are influenced by age (Ubosi et al., 1985b; Doria and Frasca, 1997), antigen type and dosage (Boa-Amponsem et al. 1998a; van der Zijpp, 1983a; Boa-Amponsem et al. 1997b; Ubosi et al. 1985a), and genetic factors (Biozzi et al. 1972; Siegel and Gross, 1980). The phenomenon of the dynamics of Ab response being under genetic control has led scientists to dissect the molecular basis of the genetic control using several dynamic parameters of primary Ab response. For example, Ab production to SRBC has been used as a parameter of Ab response competence for dissecting the molecular basis of the

genetic control underlying the dynamics of primary Ab response (Puel et al. 1995, 1996). Compared to CMI, Ab response appears to be more accessible to genetic analysis because its kinetics can easily be studied quantitatively and qualitatively by reliable parameters.

2.3.5 The association of AMI with disease resistance

The control and prevention of infectious diseases is an important issue in the animal production industry because a large portion of profits continues to be lost due to frequent outbreak of infectious diseases. For example, bird influenza has led to catastrophe in the poultry industry in certain countries, and has not been well controlled yet. Use of vaccination and antibiotics and improvement of sanitary conditions has been contributing to disease control and prevention, but applications of these means not only have a huge cost, but also bring about complications. For example, the prophylactic use of antibiotics may lead to the development of drug-resistant bacteria, which could harm humans and farm animals in the future. Such an issue has put animal production into a dilemma. Improving natural disease resistance among animals may provide a promising approach to eliminating this dilemma (Colditz et al., 1996; Lillehoj, 1994).

Results from several studies suggest that animal natural resistance to infectious diseases is associated with the level of Ab production (Gross et al., 1980; Siegel et al., 1982; Pitcovski et al., 1987; Yamamoto et al., 1991; Leitner et al., 1992; Pinard et al., 1993; Yonash et al., 1996). For example, disease resistance was different between lines of chickens divergently selected for high (H) and low (L) antibody titers to SRBC. The H line of chickens was more resistant to *Mycoplasma gallisepticum*, *Eimeria necatrix* and feather mites, but was more susceptible to *Escherichia coli* and *Straphylococus aureus* infections than the L line of chickens in the 3rd generation of selection (Gross et al., 1980). The subsequent observations in the 10th and 11th

generations of selection indicated that the H lines also was more resistant to *Eimeria tenella* than the L lines in both natural and controlled exposures (Martin et al, 1986). In addition, Martin et al. (1989) observed that the 12th generations of the H line of chicks were more resistant to a natural exposure to Marek's disease than the L line of chicks.

Several genetic factors influencing both Ab response and disease resistance have been identified in the chicken. The MHC, the B complex in the chicken, is composed of heterodimeric transmembrane glycoproteins that are essential in the presentation of antigens to T lymphocytes (Kroemer et al., 1990; Plachy et al., 1992). The MHC molecules can be divided into class I (*B-F*), class II (*B-L*), and class IV (*B-G*) in the chicken. The MHC system has been associated with antibody response (Dunnington et al., 1984, 1996; Kean et al. 1994a; van der Zijpp et al., 1987; Loudovaris et al., 1996). This is suggested by the difference in allelic frequencies for the B system between H and L lines. It was shown that B^{21} haplotype was dominant in the H line, while B^{13} haplotype was near-fixed in the L line. However, it is evident that although there is a genetic association between the B system and antibody production to SRBC, the situation is complex. This was indicated by a replicated selection experiment for anti-SRBC response. In this experiment, changes in gene frequencies of B^{21} were only observed in one of the H replicates after five generations of selection; similar situations were also observed in the L replicates.

Evidence shows that the MHC is also closely associated with resistance or susceptibility differences to a number of diseases in the chicken (Lamont, 1998). Comprehensive reviews of the MHC linkage with differences in disease resistance to different classes of pathogens have been given in several papers: resistance to viral infection (Bumstead, 1998; Gavora and Spencer, 1979; Kaufman et al., 1992; Longenecker et al., 1977; Pinard et al. 1993; White et al. 1994; Yoo and Sheldon, 1992), resistance to bacterial infections (Cotter et al., 1992; Guillot et al., 1995;

Siegel et al., 1993), and resistance to protozoal infection (Caron et al., 1997; Lillehoj, 1994; Uni et al., 1995). The most explicit case regarding the linkage of MHC and disease resistance or susceptibility is that the difference in resistance to Marek's disease virus (MDV) between MHC haplotypes is related to the level of class I molecule expression rather than its specificity (Kaufman et al., 1996). Chicken strains carrying MHC haplotypes that express fewer class I molecules, such as B^{21} strains, are resistant to MDV, while those that abundantly express class I molecules, such as B^{19}, are most susceptible (Gavora and Spencer, 1979).

Class IV MHC has been exclusively reported in avian species, as has *Rfp-Y*, the MHC-linked gene that is recognized by DNA restriction fragment patterns (*Rfp*) of the MHC class I, II and IV genes in one MHC haplotype (Briles et al., 1993; Miller et al., 1994). Although the location of the gene is within the MHC gene regions, its function is not clear. Compared to the class I, II and IV gene regions, only limited *rfp-Y* polymorphisms have been identified. However, *rfp-Y* may be associated with Ab response, since it was observed that one of the three polymorphisms of *rfp-Y* occurred in significantly different frequencies between lines divergently selected for a multitrait index that contained a parameter for Ab response (Lakshmanan and Lamont, 1998). *Rfp* polymorphisms have been reported to be correlated with differences in resistance to pathogens, especially MDV (Lakshmanan and Lamont, 1998; Wakenell et al., 1996).

Antibody response also is influenced by immunoglobulin genes (Butler, 1998; Rees and Nordskog, 1981). Immunoglobulins are membrane receptor proteins on the B cell membrane or are secreted into circulation and onto mucosal surfaces by plasma cells as antibodies. The diversity and the amount of immunoglobulins are important factors in resistance and susceptibility to infectious pathogens (Butler, 1998). Therefore, immunoglobulin genes link Ab response with disease resistance.

Cytokines also play important roles in both Ab response and disease resistance. T_h2 is one of the two main cytokine families produced by the helper CD4+ T cells (Infante-Duarte and Kamaradt, 1999; Lunney, 1998; Street and Mosmann, 1991). The T_h2 cytokines such as interleukins-4, -5, -6 and -10 stimulate the Ab response (Infante-Duarte and Kamaradt, 1999). The differentiation of a progenitor T helper cell into the T_h2 helper cell has a critical impact on the pattern of immune response and subsequently on disease development (Finkelman et al., 1997; Holdsworth et al., 1999).

Finally, the endogenous viral (*ev*) genes may contribute to both Ab response and disease resistance. It was found that *ev6* and *ev9* differ in frequency between lines divergently selected for a multitrait index containing a parameter for Ab response (Lamont et al., 1992), and both were at lower frequency in the lines selected for high immunoresponsiveness. The *ev* genes may express viral envelope glycoproteins like *gp85*, which block the receptor sites evoking resistance against exogenous infection with the avian leukosis virus (ALV) subtype that needs the specific receptor (Smith et al., 1991). Such receptor saturation or blockage also limits the congenital transmission of the virus (Smith et al., 1990). For instance, the *ev6*-carrying chicken lines express ALV-E envelope glycoproteins, and so are resistant to ALV subtype E infection (Smith et al., 1990).

In addition to natural disease resistance, immune responses including AMI also play important roles in other biological phenomena including aging and longevity (Doria and Frasca, 1997, 2000), allergy/hypersensitivity (Blumenthal, 2004), reactions to transplantation (Sims et al., 2001), autoimmunity (Turesson, 2004), stress/neuroendocrine responses (Petrovsky, 2001; Dhabhar, 2002) and animal production (Pinard-van der Laan, 2002). The importance of immune

responses has driven efforts to more fully describe and to understand the molecular basis of vertebrate immune responses.

2.4 Chicken resource populations for QTL analysis

2.4.1 Quantitative trait locus

Most economically important phenotypes, including production traits and complex genetic diseases, comprise a continuous distribution of measurable characters. These phenotypes are called quantitative traits by geneticists. In contrast to Mendelian (single-gene) traits, quantitative traits are typically affected by multiple loci or genes called quantitative trait loci (QTLs). Identification of QTLs can help us to understand the genetic control of quantitative traits underlain by the QTLs. However, it is well demonstrated that it is challenging to locate QTLs and estimate QTL effects. This is because quantitative traits can be influenced by environmental and genetic noise in addition to the polygenic mode of inheritance (Andersson and Georges, 2004). A variety of QTL mapping methods have been invented aiming to meet this challenge. QTL mapping means identification of QTL location and estimation of QTL effects with known genetic markers or genes based on demonstration of statistical significance. However, use of appropriate resource populations is a prerequisite for QTL mapping. Crosses derived from inbred lines and divergently selected lines are the most commonly used resource populations for QTL mapping.

2.4.2 The chicken as a model for QTL analysis in vertebrates

The chicken has long been an ideal and tractable model for genetic studies of biological questions of broad implications. Our understanding of the mechanisms of inheritance was significantly advanced by the classic work of Bateson in the chicken. Use of the chicken as a

model species also has extended beyond these genetic studies. Chicken has emerged as a model for QTL analysis in vertebrates. This is mainly because of the relative ease of developing appropriate resource populations for QTL analysis. Populations with wide phenotypic or genetic variation are critical to the success of QTL analysis. Long-term artificial selection has developed many breeds and experimental lines of chickens with highly varied traits, such as the meat-type and egg-type strains. No other agricultural animal has been phenotyped and selected as intensively as the chicken. Correspondingly, the list of quantitative traits of interest in the chicken extends from production characteristics, such as growth, meat quality, and egg production, to other traits such as behavioral and immune-related traits because of concern for animal health, the environment and disease resistance (Burt, 2002). The chicken also is unique among major agricultural species in that a number of highly inbred lines are available for many traits for which inbred mouse lines also exist.

The high-resolution chicken genetic map also provides an advantage for using the chicken as an animal model for QTL analysis. The current chicken genetic linkage map, initiated early in the last century (Hutt, 1949), now has 2,499 genetic markers with a total coverage of about 4,718 centimorgan (updated 11/2005, Bitgood and Somes, 1993; Groenen et al., 2000). This advantage is further enhanced with the release of the chicken genomic DNA sequence (International Chicken Genome Sequencing Consortium, 2004). Though only a draft, the chicken sequence provides a resource for rapidly moving from candidate marker to a candidate gene in QTL analysis.

In addition, the chicken reproduces rapidly and has large brood sizes, enabling several generations of large families to be generated in a reasonable time frame. The chicken, therefore, provides an ideal model for genetic mapping and QTL analysis among agricultural animals.

Various resource populations of chickens, including inbred lines and divergently selected lines, have been developed for QTL analysis.

2.4.3 Inbred lines versus divergently selected lines

2.4.3.1 Inbred lines

Inbred lines are animals of a particular species that are nearly identical to each other in genotype due to long-term inbreeding. Twenty generations of brother-sister mating will lead to lines that are roughly 98% genetically identical. Usually, such lines are considered as inbred lines. The advantages of using inbred lines include simple bi-allelic segregation at both QTL and genetic markers, full information about the linkage phase of genes at the marker loci and QTL, and versatility of the experimental design for both detection of marker-QTL linkage and estimation of genetic parameters defining genetic effects at the QTL (Soller et al., 1976). The basic experimental designs for QTL mapping that are appropriate for crosses between inbred lines include those with generations of backcross, test cross, F_2 and recombinant inbred lines (Weller, 2001).

Inbred lines not only can be used for QTL mapping in backcross and F_2 designs through linkage analysis that provides rough information about QTL location, they also can be used for QTL fine-mapping through linkage disequilibrium (LD) analysis using recombinant inbred lines (RILs) and near-isogenic lines (NILs) that are derived from inbred lines. An recombinant inbred line is derived from two inbred lines by crossing the lines and subsequent repeated selfing or sibling mating (Singer et al., 2004; Nadeau et al., 2000; Bailey, 1981; Taylor et al., 1977). Its genome is mosaic of the parental genomes. The higher density of breakpoints in RILs makes high resolution of QTL mapping possible (Broman, 2005). Other advantages from RILs include the option to propagate them eternally as inbred lines, genotyping for each strain needs to be

done only once, and phenotypic data from multiple individuals can be used to reduce individual, environmental, and measurement variability. In general, each RIL has several genomic fragments introgressed from parental lines, and both parental genotypes have equal chance on average to be contained in the introgressed fragments. The multiple introgressions in each RIL make genetic interactions between loci detectable. However, the genetic interactions may reduce the power to detect QTL. This situation could become worse if recombination frequencies are unequal throughout the genome, and lethality or reduced fitness of particular genotypes lead to segregation distortions. Moreover, QTLs with small additive effects may not be detected because they could be masked by QTLs with large effects (Gupta, 2002; Ungerer et al., 2003). However, the limitations in QTL mapping analysis with RILs caused by the masking effects of major QTL and the epistatic interactions of multiple QTL can be greatly overcome by using NIL populations.

A NIL is developed through repeated backcrossing and extensive genotyping (Keurentjes et al., 2007). Such populations consist of lines that have a single or a small number of introgression fragments in the genome. These fragments are resulted from introgressing a donor genome into an otherwise homogeneous genetic background (Brouwer and St. Clair, 2004; Caballero et al., 1995). While NILs and RILs are both immortal experimental populations and share some advantages, there are differences between them. In contrast to RILs, the presence of a single introgression segment in each NIL can increase the power to detect small effect QTL (Kaeppler, 1997). However, NILs do not accommodate testing for genetic interactions because most of the genetic background is identical within NIL populations. Therefore, NILs and RILs are relatively complementary to each other in QTL mapping analysis.

2.4.3.2 Divergently selected lines

Inbred lines are widely used in the mouse and rat, but rarely used in other vertebrate animals due to infeasibility of development of inbred lines. In the chicken, genetic lines selected for specific traits frequently are used for QTL identification, although several inbred lines of chicken have been used for identification of genes influencing resistance to avian tumors (Bacon, 2000).

Development of divergently selected lines mostly starts from a non-inbred base or founding population in which individuals share the same genetic background, followed by a programmed selection and breeding scheme where repeated assortative matings are applied and inbreeding is generally restricted. Long-term divergent selection for a given quantitative trait would lead to a significant phenotypic difference between the selection lines (Bunger et al., 2001). The phenotypic divergence could be explained by the progressive accumulation, in each line, of multiple genes endowed with coherent additive effects on the selected trait of interest. When the limit of response to selection is reached, the relevant genes can be considered to be fixed at homozygosity (Andersson and Georges, 2004; Siegel et al., 2006). Therefore, QTLs associated with the selected lines could be identified directly based on allelic frequency differences between divergently selected lines (Keightley et al., 1993). However, the frequency differences alone should not be considered a definite proof of a QTL identification because such differences are only suggestive of changing frequencies of alleles of the QTLs as a result of selection. Other factors such as genetic drift and founder effects also can contribute to allele frequency changes (Goodnight, 1988). Fortunately, the putative QTLs can be verified by the use of segregating populations derived from the divergently selected lines, such as backcross and intercross (F_2) populations.

Although divergently selected lines that orginiate from a non-inbred population have the limitations mentioned above, their unique characteristics make them superior to inbred lines in some aspects. For example, some QTLs for a trait may be not polymorphic between inbred lines used for QTL mapping because inbreeding can dramatically decrease DNA polymorphism (Jain and Jain, 1969). These QTLs cannot be identified using map-based approaches because allelic polymorphism is a prerequisite for map-based QTL identification. The chance for a QTL being polymorphic between inbred lines depends on the phenotypic difference of the trait of interest between the inbred lines used. Moreover, even if a QTL is polymorphic between inbred lines, it may still not be identified. This is because many alleles could have no or marginal differences in their effects on the trait of interest due to synonymous or non-critical mutations (Duret, 2002; Ohta and Ina, 1995). Inbred lines may not differ at marker loci. In contrast, the divergently selected lines generally do not have these problems. In addition, other relevant advantages from the divergently selected lines include the usefulness in estimating the number of QTLs for a trait and evaluating the correlated selection responses.

2.4.4 Genetic lines divergently selected for anti-SRBC

Divergent selection for Ab production to a variety of antigens has been performed in many species of vertebrate animals, but mainly in the mouse and the chicken. Sheep red blood cell is one of the most frequently used antigens for the following reasons. First, SRBC is a non-pathogenic antigen. Environmental microbes do not interfere with measurement of Ab response to SRBC. Second, SRBC is a multi-determinant and strongly immunogenic antigen (Fukuda, 1993; Chasis and Mohandas, 1992; Lowe, 2001; Cartron et al., 1998). The main reason for choosing SRBC as the selected antigen is that background genes, which are not linked to immune response genes at MHC and *Igh* loci, were shown to contribute to Ab production (Peul

et al., 1995, 1996). Third, the methods used to measure Ab levels and to detect the specific Ab-producing cells in the SRBC-triggered immune response have been established. The parameters of the response are also well documented. Lastly, selection for Ab production to SRBC has been considered an attractive approach to improve general enhanced disease resistance to multiple pathogens (Gross et al., 1980). Among the lines divergently selected for Ab production to SRBC, the classic ones are the Biozzi lines of mouse and the Siegel lines of chicken (Biozzi et al., 1972; Siegel and Gross, 1980).

The Biozzi lines of mouse were initiated from a founding population of outbred albino mouse (Biozzi et al. 1972, Feingold et al. 1976). Based on anti-SRBC Ab titers, mice with the extreme phenotypes, in upward or downward directions, were selected to make assortative matings. This selection for H and L anti-SRBC was repeated for successive generations until maximal divergence of the two lines was achieved. As the results of the successive selections, the intensity of Ab response became persistent in the L line by the 14^{th} generation and in the H line by the 18^{th} generation. Also, the range of individual variability decreased progressively throughout the selective breeding (Biozzi et al., 1972). The interline separation appeared to have reached its maximal extent around the 16^{th}-18^{th} generations, which clearly indicates that Ab production is determined by the cumulative effects of several quantitative genes. Moreover, the big change in frequency distribution of anti-SRBC titers from the starting population to the 20^{th} generation of the mouse population suggested a high degree of homogeneity has been achieved. Further, it implies that all the genes responsible for Ab production have segregated in each line by the 20^{th} generation and can be considered homozygous. In addition, the heritability (h^2) of the trait was between 0.18 and 0.36 in the 14^{th} -17^{th} generations. The number of loci that regulate the quantitative Ab response was estimated to be about 10 loci (Feingold et al., 1976).

The same selection strategy was applied to the chicken. The Cornell Randombred White Leghorn population (King et al., 1959) was the base stock. Innoculations of 0.1mL of a 0.25% suspension of SRBC via the brachial vein were administrated when chicks were between 41 and 51 days of age. Antibody titers to SRBC were determined 5 days post-inoculation using hemagglutination procedures, with titers expressed as $\log_2$ of the reciprocal of the highest dilution in which there was hemagglutination. It was shown that response to selection was immediate (Siegel and Gross, 1980). Frequency distributions of Ab titers in the divergent lines changed dramatically across generations (Martin et al., 1990). Bimodality, which is evident in both lines through the 10^{th} generation, almost disappeared by the 14^{th} generation, although there was still minor overlap in frequency distributions of the lines. Realized heritabilities for the 10^{th} generation through 14^{th} generation were 0.25 and 0.23 for high and low antibody response, respectively.

2.5 Approaches to identification of QTLs

Detection of QTLs has become one of the most popular topics since the late 1980s, although the basic principles and methodology have been around since the 1920s, almost immediately after the demonstration of the chromosomal theory of inheritance and Fisher's polygenic theory of quantitative variance. This delay was mainly due to the lack of segregating genetic markers in species of interest. Before 1980, the genetic markers available were related to morphology, blood groups, and biochemical polymorphisms. These were insufficient to provide complete genome coverage. In addition, most markers were bi-allelic with one allele predominating in the population, and many displayed complete dominance. These markers were not optimal for QTL detection. With the advent of DNA-level genetic markers in the early 1980s,

and especially microsatellite markers in the 1990s, the problem with limited availability of suitable genetic markers has been resolved. It is now clear that a genetic map saturated with polymorphic codominant Mendelian markers can be generated for almost any species. Currently, a map-based approach, as one of structural genomics-based approaches for QTL identification, has become routine. Although these classic approaches continue to play an important role in QTL identification, functional genomics and comparative/*in silico* genomics-based approaches have emerged and have been demonstrated to be efficient in QTL analysis. The following sections will illustrate these approaches.

2.5.1 Structural genomics-based approaches

Structural genomics refers to the analysis of the constitution and structure of chromosomes rather than proteins. As a branch of genomics, structural genomics has an indisputable role in biological research. Although structural genomics has reached the level of whole genome sequencing, genetic markers and genetic mapping, which were developed at an earlier stage of structural genomics, are still important components of structural genomics. They have important implications in QTL analysis.

2.5.1.1 Genetic markers

A genetic DNA marker is a segment of DNA that can be identified and tracked. A marker can be a gene, or it can be some section of DNA with an unknown function. The properties of a genetic marker include locus specificity, polymorphism in the studied population, and easy genotyping. The quality of a genetic marker is typically measured by its level of heterozygosity in the given population and polymorphism information content (Botstein et al., 1980).

There are several criteria for categorizing genetic markers. First, there are three types of markers based on location, Types I, II and III (Groenen et al., 2000; Weber and May, 1989;

O'Brien et al., 1999). Type I markers are within coding regions of genes, such as some restriction fragment length polymorphisms (RFLPs). Type II markers are within noncoding DNA sequences, such as microsatellites or simple sequence repeats (SSR, Weber and May, 1989). Type III markers are within either coding or noncoding DNA sequences, such as single nucleotide polymorphisms (SNPs, O'Brien et al., 1999). In the chicken, the number of the Type I markers is approximately 350 (Groenen et al., 2000). In the past, Type I markers were not thought to have extensive applications in QTL mapping, due to the laborious RFLP analysis and the limited number of RFLPs that were observed within Type I loci. However, with the isolation of almost 400,000 expressed sequence tags (ESTs) and the identification of novel genetic polymorphisms in these genes, Type I markers are expected to have a wider application in chicken genomics and QTL identification than they had in the past. In contrast, Type II markers have received considerably more attention than Type I markers, and have been the marker of choice for genetic mapping and QTL searches. This is because Type II markers are highly polymorphic and are easily genotyped by PCR-based assays. As Type III markers, SNPs are the most common form of genetic variation in the genome (Wang et al., 1998; Dawson, 1999). High-resolution genetic maps based on SNPs are currently under development for several of the human chromosomes (Mullikin et al., 2000; Taillon-Miller and Kwok, 2000). SNPs are excellent genetic markers for mapping studies.

The second criterion for categorizing genetic markers is based on whether there is prior knowledge about the marker sequence (Dodgson et al., 1997; Weber and May, 1989). In this scheme, genetic markers can be classified as either clone/sequence-based or fingerprint markers. The fingerprint markers do not require prior knowledge of the marker sequence and include random amplified polymorphic DNA (RAPD, Williams et al., 1990), amplified fragment length

polymorphisms (AFLP, Vos et al., 1995), and minisatellites or variable number of tandom repeats (VNTR, Jeffreys et al., 1985). In contrast, the clone/sequence-based markers either require the isolation of a cloned DNA fragment or require marker sequence determination and include SSRs, RFLPs, sequence-tagged sites (STSs), and ESTs.

Last, based on the type of information genetic markers provide, genetic markers can be divided into three categories: the biallelic dominant markers such as RAPDs and AFLPs, the biallelic codominant markers such as RFLPs and SNPs, and the multiallelic codominant markers such as VNTRs and SSRs (Dodgson et al., 1997; Weber and May, 1989).

In the following paragraphs, only RAPD, RFLP, SSR and SNP markers will only be described in detail and other genetic markers will be omitted due to less involvement in this dissertation research and less frequencies of use.

Random amplified polymorphic DNA is synthesized in a polymerase chain reaction (PCR) with a single arbitrary primer (8- to 12-mer oligonucleotide; Williams et al., 1990). The annealing temperature is generally lower for RAPD than for regular PCR. A spectrum of DNA fragments may be generated with an arbitrary primer. The resulting fragments can easily be separated on an agarose gel by electrophoresis. Among them, one or more fragments may be polymorphic and can be genetically mapped.

The advantages of RAPD are its simplicity, rapidity, and ability to generate numerous polymorphisms (Chen and Leibenguth, 1995). RAPDs are also fairly evenly distributed over the genome (Levin et al., 1994; Chen and Leibenguth, 1995). Therefore, it has been a powerful technique for genetic analysis (Chapco et al., 1992; Kiss et al., 1993; Landry et al., 1993; Wight et al., 1993; Williams et al., 1990). There is evidence indicating that RAPD technique is a useful tool for identifying putative QTLs at genome-wide scale. For example, Martin et al. (1991) and

Gu et al. (1998) used the technique to identify markers linked to a *Pseudomonas* resistant gene in the tomato and a RAPD-derived marker linked to progressive rod-cone degeneration in dogs, respectively.

The advantage of this method is attributable in part to the short length and arbitrariness of primer used in the RAPD-PCR. The major limitation of RAPDs is their dependence on the exact PCR conditions applied. Reproducibility problems may arise from this dependence (Dodgson et al., 1997). Another shortcoming of RAPD markers is that they rely on bi-allelic dominance, which reduces their efficiency in population genetic studies (Lewis and Snow, 1992; Lynch and Milligan, 1994; Harris, 1999). However, these drawbacks of RAPD markers can be overcome by converting them into sequence characterized amplified regions (SCARs) because the SCARs can be amplified with site-specific primers that are designed using RAPD marker sequences (Martin et al., 1991; Gu et al., 1998).

Restriction fragment length polymorphism (RFLP) markers were the first generation of DNA markers for different organisms including human, chicken, turkey and bovine (Strachan and Read, 1999; Vignal et al., 2002; Weissenbach, 1998). RFLPs can result either from a point mutation creating or destroying a restriction site or from insertions or deletions altering the length of a given restriction fragment. RFLPs are time- and labor- consuming markers. However, this shortcoming is now becoming less problematic because the combination of PCR with RFLP greatly simplifies RFLP technique. In a PCR-RFLP, a sequence containing the variable restriction site is amplified by PCR, the amplicon is then subject to digestion with restriction endonucleases, and the restriction pattern of the amplicon is visualized by electrophoresis on a gel (Strachan and Read, 1999). In addition, the fundamental limitation of RFLP markers is their low informativeness because RFLPs are generally bi-allelic codominant markers.

The second generation of DNA marker-based maps involved simple sequence repeats (SSRs). Microsatellites are loci present in nuclear DNA that consist of repeating units of 1-6 base pairs in length (Turnpenny and Ellard, 2005). The repeating units can occur 5 to 100 times (Litt and Luty, 1989; Tautz, 1989; Weber and May, 1989; Dodgson et al., 1997). Microsatellites owe their variability to an increased rate of mutation compared to other selectively neutral regions of DNA. These high rates of mutation can be explained most frequently by slipped strand mispairing (slippage) during DNA replication on a single DNA double helix. Mutation also may occur during recombination during meiosis (Blouin et al., 1996; Hughes et al., 1993).

Microsatellite markers often present high levels of inter- and intra-species polymorphisms, particularly when tandem repeats number is ten or greater (Queller et al., 1993). Microsatellites have been referred to as the second generation of DNA markers for gene-mapping studies because they are typically selectively neutral, co-dominant, and highly polymorphic. At least 959 microsatellites have been recorded on the chicken consensus map (update 2005, Groenen et al., 2000). The density of genetic markers identified for the chicken is sufficient to allow genome-wide screening for QTL in well-defined, large, family-pedigreed experimental populations. In addition, a microsatellite locus usually have multiple alleles. Thus, genotypes within pedigrees are often informative, and a particular allele could be traced back to its progenitor. This is the reason why microsatellites are frequently used in population genetic studies, paternity determination, and recombination mapping. Microsatellites can be amplified with primers for their flanking regions using PCR. The flanking sequences are obtained from cloning and subsequent sequencing of DNA segments containing microsatellites. Although microsatellites are versatile molecular markers, performing high-throughput genotyping for

microsatellites, as for SNPs, seems infeasible. This may restrict the use of microsatellites in large-scale research.

Single nucleotide polymorphisms arise from single nucleotide substitutions (transitions are more frequent than transversions) or single nucleotide insertions/deletions. SNPs can occur in genes (promoter, exons or introns) and intergenic regions. The SNPs within the coding sequences are categorized as either synonymous (does not result in an amino acid change) or non-synonymous (results in an amino acid change). Non-synonymous SNPs are of interest due to their potential effects on protein expression and, ultimately, the phenotype. About 26 to 32% of non-synonymous SNPs are predicted to affect protein function (Chasman and Adams, 2001). In contrast, synonymous SNPs probably have minimal effects on gene expression. However, both synonymous and non-synonymous SNPs are valuable tools as genetic markers in mapping studies.

Single nucleotide polymorphisms are very abundant in the genome. For instance, in humans, it has been estimated that there is one SNP per 1,000 to 2,000 bases of genomic DNA. A total of 2.8 million SNPs were identified and mapped in the chicken by the International Chicken Polymorphism Map Consortium (Wong et al., 2004). Smith et al. (2001) estimated that the frequency of SNPs in EST-targeted genomic DNA was 1 per 470 bases. This abundance combined with large-scale automation of SNP genotyping makes SNPs the third generation of genetic markers (Collins et al., 1997; Wang et al., 1998), although SNPs are less informative, like other biallelic markers. The abundance also makes fine-mapping studies and population-based studies that require linkage disequilibrium analysis possible. A high density of genetic markers is the prerequisite for linkage disequilibrium.

The distribution of SNPs in the genome can sometimes be clustered although random (Feder et al., 1996). The distribution of SNPs forms the basis of molecular haplotypes (Johnson et al., 2001). An advantage of using haplotype-tagging SNPs is that it may be possible to detect trait associations that would not otherwise be observed unless the gene were evaluated as a functional unit, rather than evaluating individual SNP markers. Identification of haplotype-tagging SNPs also would reduce the costs of genotyping, by typing for molecular haplotypes rather than individual SNPs within a gene.

Single nucleotide polymorphisms can be discovered or detected through multiple approaches. The optimal approach is to perform a comparative analysis of the DNA sequences from different individuals to identify nucleotide variations. However, this approach is laborious and expensive, and there is a chance that a SNP may not be identified within the tested individuals. Single-stranded conformational polymorphism, PCR-RFLP, melting temperature statistics (denaturing-HPLC), pyrosequencing, *in-silico* EST data mining, and microarray-based genotyping are some other options. Development of microarray-based SNP genotyping contributes to the popularity of using SNPs as genetic markers in biological research. This technique is based on oligonucleotide hybridization analysis. It allows quick and large-scale genotyping of SNPs. However, this technique can be prohibitively expensive for typical laboratories. The high cost of SNP detection often limits its use.

As discussed below, genetic markers have many applications in agriculture and biological fields. Some typical applications include generation and integration of genome maps (e.g., genetic maps); population genetics and ecological studies (e.g., genetic diversity); identification of genes underlying monogenic and polygenic traits; marker-assisted selection; parentage control and individual identification; and phylogenetics (e.g., estimation of genetic distance).

2.5.1.2 Genetic map

Generation of a genetic map is one of the most important applications of genetic markers. A genetic map or linkage map is a map of a genome that shows the relative positions rather than physical locations of the genes and/or markers on the chromosomes. A genetic map is generated based on genetic distance between markers, which is estimated from the frequencies of recombination between markers during crossover of homologous chromosomes (Collard et al., 1998). The higher the frequency of recombination between two genetic markers, the greater the physical distance between them, and *vice versa*. In other words, the chance of a crossover producing recombination between the markers is directly related to the distance between two markers. As stated in the Mendel's Second Law (the law of independent assortment), chromosomes assort randomly into gametes during meiosis. Thus, the independently assorting markers that are located on different chromosomes will have a recombination frequency of 50%. However, markers on the same chromosome are not always true for the Mendel's Second Law. If two markers that are closely located on the same chromosome, they will have a recombination frequency of less than 50% due to non-independent assortment. This is because the genetic markers are linked on the same chromosome. Genetic linkage means the likelihood for alleles of two genetic markers on the same chromosome to be inherited together is higher than that for those on different chromosomes. Genetic linkage is measured by recombination frequency, and can be calculated as the percentage of the offspring with new combinations of alleles, which are different from the combinations inherited from the parents. The tighter the linkage between markers, the lower the recombination frequency, and *vice versa*. It is also true that the tighter the linkage, the smaller the physical distance, because of the relationship of recombination frequency

with physical distance between genetic markers. In a genetic mapp, a 1% recombination frequency is equalivalent to 1 map unit (m.u.) or 1 centimorgan (cM).

Genetic maps make feasible a wide range of studies, including mapping and positional cloning of monogenic traits, linkage-based QTL analysis (or genetic dissection of polygenic traits), construction of genomic-wide physical maps, rapid marker-assisted construction of congenic strains, and evolutionary comparisons (Copeland et al., 1993a,b).

2.5.1.3 Linkage-based QTL mapping

A genetic map is essential for linkage-based QTL analysis. The principle behind this analysis is that the inheritance pattern of a QTL can be tracked by genetic markers that are linked to the QTL (Knapp et al., 1990; Ferreira, 2004; Tanksley, 1993). This is because the linkage between a QTL and a genetic marker can be indicated by stastistically testing the association between the genetic marker and any quantitative trait influenced by the QTL. The association between the marker and phenotype is related to the recombination frequency between the QTL and the marker, which is dependent upon the distance or the degree of linkage between them. Therefore, the location of a QTL can be narrowed down by the linkage-based analysis.

Conceptually, there are three steps in the linkage-based analysis: screen the whole genome using a dense set of genetic markers; calculate linkage statistic for each marker; and identify the genomic regions indicated by linkage statistic that shows a significant deviation from what is expected from random assortment.

In practice, interval mapping is often used for linkage-based QTL analysis (Haley and Knott, 1992; Jensen, 1989). This is because single- and two- marker QTL linkage mapping are not efficient when compared to interval mapping. Single marker-based analysis cannot reveal the position and effect of a QTL except knowing there is a QTL linked to the marker (Darvasi and

Weller, 1992; Lander and Botstein, 1989). Two-marker based analysis, however, gives some information about position but too little to be useful. In contrast, interval mapping utilizes information from the markers flanking each interval, for example, every 1 cM, to evaluate support for the existence of a QTL (Haley and Knott, 1992; Jensen, 1989). Therefore, it gives more precise information about a QTL than single- or two-marker approaches. In interval mapping analysis, the maximum likelihood (ML) method is used to evaluate, for each putative QTL position, the log of the ratio of the maximum of the likelihood function under the null hypothesis of no segregating QTL to the maximum of the likelihood function under the alternative hypothesis of a QTL segregating at that position, given the marker and phenotypic data (Jansen, 1993; Kao et al., 1999). This provides a logarithm of odds (LOD) score for each putative QTL position. The putative position with the highest LOD score is the most likely position of the QTL (Weller, 1986, 2001). An alternative is to use a regression approach, which gives a high-quality approximation to the ML methods (Xu, 1995, 1998).

In a linkage-based QTL analysis, a question that remains thorny is what standard should be used for declaring linkage. This is an issue because the test statistic used in the analysis fluctuates substantially just by chance across an entire genome scan. A lax standard usually leads to a large amount of false positive linkage claims. Contrarily, if the standard is too stringent, if will be similarly risky because many false negatives may be found. Therefore, it is essential to use an appropriate standard that can minimizing these two risks, and the assertions in a linkage analysis will stand the test of time. However, meeting this need requires both a mathematical understanding of how often positive results will occur just by chance and a value judgment about the relative costs of false positives and false negatives (Chen and Storey, 2006; Dalmasso et al., 2005).

The importance of distinguishing between point-wise significance levels and genome-wide significance levels is another important issue in linkage-based QTL analysis. The point-wise significance level is the probability that an extreme deviation would occur at a specific locus just by chance. The genome-wide significance level is the probability that a deviation would happen somewhere in a whole genome. The former concerns a single test of the null hypothesis of no linkage; the latter involves fishing over a large number of tests to find the most significant result. There are three techniques to solve the problem related to multiple tests in the interval mapping, namely, Bonferroni correction, permutation tests, and use of the false discovery rate (FDR).

For a linkage-based QTL analysis, the power to detect and locate QTLs depends on: 1) the ability of the analysis to separate the QTL effect from environmental and other effects, which determines the accuracy with which QTL effects are estimated; 2) the number of recombination events in the resource population. The structure of the resource population has an important influence on the power of QTL detection. Studies on humans, large animals, and trees have used existing populations (Diez-Tascon et al., 2001; Luo, 1998; Spelman, et al., 1996). Segregating populations in the existing populations are sought by examining the numbers of full-sib or half-sib families and by use of the grand-daughter population in the grand-daughter design, for example. In comparison, studies on small animals such as chickens and mice for which selection is feasible usually use inbred lines and divergent lines. These lines are used to generate segregating populations such as backcross, F_2, test cross, and recombinant inbred lines. The F_2 and backcross designs have led to successful QTL mapping in a number of mammalian species.

QTL detection experiments are expensive. The cost of genotyping is often the largest cost in the experiment. Therefore, strategies that either reduce the number of genotypings or increase

the power to detect QTLs with the same amount of genotyping information have been proposed. These strategies include selective genotyping, selective DNA pooling, and sequential sampling.

Selective genotyping is a method of QTL mapping in which the analysis of linkage between markers and QTLs is carried out by genotyping only individuals from the high and low tails of the phenotypic trait distribution in the population (Darvasi and Soller, 1992). In half-sib designs, the selective genotyping usually is done within each sire family. Individuals deviating the most from the mean are selected for genotyping. They are more informative than those with average phenotypes. This is because QTL genotype can be more clearly inferred from phenotype in these individuals.

In the selective pooling technique, linkage between a marker and a QTL is determined based on allele frequency difference at the marker between the pooled DNA samples of the extreme high and low phenotypic groups of offspring (Darvasi and Soller, 1994; Lipkin et al., 1998). In other words, if the genetic marker is linked to a QTL affecting the quantitative trait, one allele at the marker loci will have a higher frequency in the high tail, and the alternative allele will have higher frequency in the low tail. This technique has several difficulties that are needed to overcome, such as accurate quantification of individual DNA samples for making the pooled DNA samples, and removal of 'shadow' bands when segregating microsatellite markers by electropherosis (Darvasi and Soller, 1994; Lipkin et al., 1998). In addition, this technique only has the power to detect QTLs affecting the trait, for which the pooled DNA samples were made. The power to detect QTLs underlying other traits will be much lower, unless there is a high genetic correlation between the traits. In practice, the pooled DNA samples must be replicated to increase the accuracy of results, which increases the number of genotypings required.

In the sequential sampling technique, a small sample is genotyped at the outset rather than a sample large enough to obtain a desired statistical power for all markers in the preliminary step (Motro and Soller, 1993). Further genotyping is not done for those markers that either clearly show significant or non-significant effect. Additional individuals will be genotyped only for those markers that display marginal significance. By this method, it is possible to reduce by nearly half the total number of genotypings of a single trait required.

In the linkage-based QTL analysis, the researcher should be aware of several issues. First, a QTL may not be a gene. This is suggested by recent analysis of mouse genome. For the mouse, the coding part of the genome is relatively small (~1.5% of the genome, Waterston et al., 2002). However, the latest estimates indicated that about 5% of the mouse genome is under selection on average (Waterston et al., 2002). This implies that there are many non-coding sequences under selective control. Provided this, it appears that many QTLs are likely to located outside of the coding regions across the genome. An example is the callipyge trait in sheep, probably caused by a long-range control element within the *DLK-GTL2* imprinted domain (Freking et al., 2002; Georges et al., 2003). A second area of concern is that a genetic effect in a chromosomal region rather than a single gene is mapped. The chromosomal region may consist of many linked genes. Therefore, a single, large effect locus might contain several small-effect genes or genes that have opposing effects on a phenotype, and the linkage may break up in subsequent generations. Third, a QTL may be imprinted and therefore may not be detected by assuming Mendelian inheritance. Fourth, a particular effect may be overestimated since the QTL effect is made up of both a true effect and environmental error, and the QTLs which exceed the significance threshold are more likely to have received a favorable environmental error component. Georges et al. (1995) demonstrated that the overestimation of QTL effects is more evident for QTLs with small effects

and for experiments with smaller numbers of animals, using simulation. Last, QTL detection experiments do not detect epistatic interaction between loci.

Finally, it is well-known that linkage-based mapping of QTL is a rather crude tool (Devlin and Risch, 1995). On average, a 10 cM chromosomal segment will have about 200 genes, and 10^7 base pairs. Although it would seem that increasing the density of markers genotyped should increase the resolution of a QTL's location, this is true only up to a point. For most practical situations, reducing marker spacing below 20 cM does not increase QTL resolution (Darvasi et al., 1993). This is because the accuracy of locating a QTL depends on the frequency of recombination observable in the genotyped progeny. Linkage analysis requires a large number of progeny to position QTLs with any precision; such numbers are often beyond the reproductive capacity of the target species or resources of the experimenter. The selective phenotyping technique may increase mapping accuracy marginally. The rationale for selective phenotyping is that once a QTL is mapped to a given interval, only recombinant individuals within that interval contribute to further mapping accuracy. Thus, the total number of phenotypes determined can be reduced by only phenotyping progeny with recombinations within the confidence interval. In comparison, linkage disequilibrium-based approaches which will be discussed in the following sections are efficient for QTL fine mapping.

2.5.1.4 Linkage disequilibrium

Linkage disequilibrium (LD), also known as allelic association, is defined as the non-random association of alleles between two loci (Hendrick, 2000). It means that two alleles at adjacent loci are inherited together more often than the expected when they were segregated independently in the population. This definition does not help illustrate the distinction between linkage and LD mapping. In fact, LD between a marker and a QTL is required if the QTL is to be

detected in either sort of analysis. Even so, it is not hard to distinguish linkage analysis from LD analysis. Linkage analysis only considers the LD existing within families or within an F_2 population and broken down by crossover after only a few generations. Therefore, only the recent recombinations in the population are used in linkage analysis, and the LD may extend for tens of cM. In contrast, LD mapping requires a marker allele to be in LD with a QTL allele across the entire population through many generations. To be a property of the whole population, the association must have persisted for a considerable number of generations, so the marker(s) and QTLs must be closely linked. LD mapping uses the historical recombinations in the population.

Because the classic definition is not particularly illuminating with respect to the causes of LD, Hayes et al. (2003) introduced an alternative multilocus definition, in which LD is defined as the chromosome segment identity. In this definition, an ancestor's chromosomes are broken down by recombinations in each generation, and as a result, only small regions on the chromosomes of current descendants of the ancestor can be traced back to the chromosomes of the common ancestor after many generations. These chromosomal regions are therefore identical by descent (IBD). In other words, the alleles at different loci on these regions are in LD. Many factors may cause LD, including migration, genetic drift, mutation, selection, small effective population size, or other genetic events that the population experiences (Lander and Schork, 1994; Hedrick, 2000). For example, in human and livestock populations, small effective population size is generally implicated as a key cause of LD. Selection is another important cause of LD. Selection is likely to have a stronger impact on LD around specific genes than that on other genomic regions.

2.5.1.5 LD-based mapping

While linkage analysis still has a part to play to meet our needs for QTL identification, a shift from linkage analysis to association study has taken place for several reasons (Reich et al., 2001). First, genetic variation with small or moderate effects on phenotypes is more likely to be detected by LD-based association studies than by linkage analysis, as noted by Risch and Merikangas (1996). Second, LD analysis provides a useful approach for QTL fine mapping. Small segments of chromosome descended from the same common ancestor in the current population, implied by existence of LD, is an important factor in QTL fine mapping. In addition, if LD contains genetic variants within a gene, a genome-wide LD analysis will provide extra benefits to QTL mapping. This is because variants with known biological functions can be examined in such a LD analysis, and the number of genes that can be examined generally is not an issue. Last, LD has the advantage of reducing the number of genotyped variants because variants in strong LD are likely to be inherited together and one can use a subset of tagging markers as proxies for the entire set.

2.5.1.5.1 LD-based association study

Genetic association studies are used to determine the association between a genetic marker and a disease or trait. If the association is present, a particular allele of the marker will be more likely to exist in an individual carrying the trait than expected by chance. The major designs for LD-based association studies include case-control design and family-based design (or transmission disequilibrium test, TDT) (Weller, 2001).

Case-control studies are a classical tool for studying associations between genes/markers and phenotypes such as a disease, trait, or other condition (Risch and Teng, 1998; Teng and Risch, 1999; Chen et al., 2004). In case-control studies, the cases are those who have the disease

or trait under study, and the controls are those who are either unaffected or randomly selected from the population (Kirkwood, 1988). The allele or genotype frequency of a marker then is compared between the cases and controls. If there is a difference in the allele or genotype frequency of the marker between the two groups, it indicates that the variation increasing the likelihood of the trait may be associated with the genetic marker. One problem with the case-control design is that allele and genotype frequencies vary between populations under different conditions. If the case and control populations are not well identified for the conditions, then the confounding effects of population stratification may lead to a false positive association. In other words, a phenotype-marker association can arise simply as a consequence of population structure, rather than as a consequence of linkage or LD.

The potential confounding effects caused by population stratification could be avoided in a family-based association study. In this study, the parents are used as controls for the case that is their affected offspring (Mitchell, 2000; Deng and Chen, 2001). The most commonly used test is the TDT. The TDT compares the number of times a marker allele is transmitted versus not transmitted from a parent heterozygote for the marker to an affected offspring. Under the hypothesis of no linkage or LD, these values should be equal. In other words, there is an equal chance for the alleles of the genetic marker under test to be transmitted from a heterozygous parent to the offspring. This null hypothesis can be tested by the Chi-square test (Mitchell, 2000; Deng and Chen, 2001).

2.5.1.5.2 LD-based QTL fine mapping

LD has been successfully used for mapping genes underlying genetic disorders in association studies (Hastbacka et al., 1992; Kestila et al., 1994; Lehesjoki et al., 1993; Kaplan et al., 1995). In addition to association studies, however, LD also has an important role in QTL fine

mapping, especially for those traits with continuous distributions of phenotypes (Xiong and Guo, 1997).

LD-based QTL fine mapping involves the use of different types of populations including outbred populations (e.g., the human and the cow) and structured experimental populations (e.g. the mouse). The existence of LD is the prerequisite for their use in QTL fine mapping. Mapping resolution is mainly related to the level of LD. When LD is low, high-resolution mapping is achievable. However, the extent of recombination in the population is the determining factor for the level of LD. In structured experimental populations, such as advanced intercross lines (Darvasi and Soller, 1995), recombinant inbred lines (Taylor, 1978), and near isogenic lines, the recombinations result from repeated crossings. In contrast, in outbred population, the recombinations are historically accumulated.

Methods for LD-based QTL mapping are still being developed. The most frequently used methods include identity by descent-based mapping in outcrossed populations (e.g., granddaughter design, Riquet et al., 1999) and haplotype-based LD mapping (Meuwissen and Goddard, 2000). In addition, Xiong et al. (2002) proposed phenotypic selection and haplotype LD mapping as two strategies for increasing the probability of detecting LD.

In summary, linkage and LD analyses are usually used for QTL mapping. Linkage mapping is appropriate for low resolution mapping to localize trait loci to broad chromosomal regions within a few cM, and it is based on family data. LD mapping, on the other hand, is useful in QTL fine mapping, and it is based on both population and family data (Mitchell, 2000; Deng and Chen, 2001). Discovery of SNPs provides a tremendous opportunity for multipoint fine mapping.

2.5.2 Functional genomics-based approaches

Functional genomics is a branch of genomics that studies the biological function of genes and their products (Yaspo, 2001). It utilizes a wide-ranging set of biological techniques to convert molecular information contained in a genome sequence into information on gene functions and effects. In a broad sense, functional genomics involves transcriptomics, proteomics, and metabolomics.

Biological techniques used in transcriptomics can be categorized into single gene and genome-wide techniques (Hiendleder et al., 2005). The former includes northern blotting, RNAse protection assay, reverse transcription PCR (semi-quantitative RT-PCR and quantitative RT-PCR), knock-outs, knock-ins, and tagged and untagged mutagenesis. Genome-wide techniques include differential display, representational difference analysis, suppression subtractive hybridization, serial analysis of gene expression, and DNA microarrays (Schena et al., 1998). Only DNA microarrays for gene expression profiling, which support high throughput and automation, will be described here.

2.5.2.1 Microarray-based expression profiling

Microarray-based expression profiling has developed into a method for identifying differentially expressed genes (Hardiman, 2004; Mandruzzato, 2007). The basic concepts about microarray analysis have been previously outlined by Murphy (2002). The DNA probes that are complementary to the genes of interest are immobilized on a surface at defined positions. DNA targets that are reversely transcribed from the sample transcriptome are fluorescently labelled, eluted over the immobilized surface where the probes are located, and hybridized with probes. The presence of bound DNA is detected by the fluorescence emitted from the immobilized surface using specialized scanners. The fluorescent signal is captured and converted into data

that can be analyzed using sophisticated software. A DNA microarray featuree generally is performed on a space of 200 microns or less in diameter. It has two basic formats, cDNA microarray and oligonucleotide microarray, based on the types of probes used. In a cDNA microarray, the probes are derived from many different types of mRNAs using reverse transcriptase, and have longer pieces of DNA than oligonucleotide probes used in an oligonucleotide microarray (Hughes et al., 2001). These cDNAs can be spotted onto the chips/glass slides using robotics, and adhered to the glass slides by covalent bonding or electrophoretic bonding (poly-L-lysine coated plates) or fixed to the glass slides by ultraviolet irradiation. In contrast, in oligonucleotide microarrays (e.g. Affymetrix array), probes are shorter pieces of DNA (Dalma-Weiszhausz et al., 2006). They are synthesized either *in situ* (on the chip) or by conventional DNA synthesis techniques and later applied to the chips. The oligonucleotides synthesized *in situ* are manufactured by light-directed chemical synthesis, which combines photolithography and solid phase DNA synthesis (Fodor et al., 1993; Pease et al., 1994). Synthesis proceeds by the use of photochemically removable protecting groups on the 5' end of a growing oligonucleotide, and a photolithographic mask is used to specify which spots are deprotected for the next round of synthesis.

2.5.2.2 Variability in microarray analysis

The underlying principle for the microarray technique is hybridizaton, which is simple to understand. Yet, the microarray technique is not simple to perform, as it is very sensitive to the influence of many variables that may lead to low reproducibility of microarray-based analysis. Therefore, it is necessary to validate the results from micoarray analysis using an alternative method, such as quantitative RT-PCR, Northern blots, or ribonuclease protection assays.

In microarray analysis, the sources of variability are: 1) biological variability, 2) variability in sample preparation (total RNA isolation as well as labeling), and 3) variability in the system (instruments and arrays, Brown et al., 2004; Chen et al., 2004; Huang et al., 2003). Among these, the system noise is negligible. This is because that standardized processes that are involved in a microarray experiment, such as hybridization, washing, staining, and scanning, as well as the quality controls in manufacturing processes generally make system noise insignificant when compared with other sources of variation (Altman, 2005; Spruill et al., 2002; Sievertzon et al., 2006). Obviously, the standardized processes include regular calibrations of all equipment used in a microarray experiment to ensure accuracy. Technical replicates usually are used to evaluate technical variation including that caused by system noise. Controlling the variability from sample preparation is the next challenge. The variability from sample preparation could be large and unnecessary. Careful technique and well-planned procedures can greatly reduce the source of variation. During sample preparation, it should be guaranteed that all RNA samples hybridized to the gene expression arrays meet assay quality standards (Dumur et al., 2004). It is necessary that all initial total RNA be examined on an agarose gel for the ribosomal RNA bands. Non-distinct ribosomal RNA bands imply poor quality of RNA sample. The poor quality generally is due to RNA degradation. The use of RNA sample with poor quality can lead to failure of a microarray experiment. A 260/280 absorbance reading is another measurement of RNA quality. The 260/280 ratios ranging from 1.8 to 2.1 are acceptable. Ratios < 1.8 suggest protein contamination. Ratios > 2.1 suggest degraded RNA and/or excess free nucleotides.

In the second step of sample preparation, reduction in process variability also helps minimize variability in microarray results. A considerable source of variation in the labeling process may come from operator-to-operator differences. Care should be taken in the reverse

transcription and labeling protocols. Consistency in these processes can reduce potential variability in a microarray experiment. Minimizing such variability could be achieved by the following common practices: 1) process all RNAs in a batch; 2) use reagents with the same lots; 3) prepare reagent master mixes; and 4) let a single scientist responsible for all the bench manipulations. However, often this is not practical, as some experiments can be quite large and occur over an extended period of time. Thus, it is important to determine and reduce variations in the process of sample preparation, both within and between the bench scientists (Dumur et al., 2004).

The last source of variability is biological variation. Unlike technical variation, biological variation is system dependent and may be more difficult to control, and sometimes be beyond control. However, there are methods for handling such variation. First, controlling as many variables as possible is the best choice. Second, if some factors are beyond control, then randomize them. When replication is sufficient, random selection can limit the impact of the uncontrollabe factor on the data. Stratification and strata-weighing of the data in the final analysis also help mitigate the impact. In addition, a pilot study is very helpful in this situation because it will give some important information for judging the design of real experiment.

It is worth noting that pooling mRNA from biological replicates can reduce the biological variability among arrays. Many investigators favor this strategy because sample size can be increased without purchasing more microarrays. However, some potential problems caused by mRNA pooling should be realized by investigators (Kerr, 2003; Churchill, 2002; Kendziorski et al., 2003, 2005). First, pooling is not always beneficial. For example, in the context of classification, pooling impairs the ability to assess inter-individual variation and covariation. Second, it is impossible to estimate variance with one pool per group. The variance is required

for inference testing. Third, even one outlier sample can yield misleading results. Finally, the measurements avaraged by sample pooling do not necessarily correspond to mathematical averages of individual measurements, although pooled sample is made from individual samples (Kendziorski et al., 2005). Nevertheless, pooling is beneficial if the only goal of a microarray experiment is to identifydifferentially expressed genes. It is ture especially when biological variation is higher than measurement error, and when biological samples are not expensive compared with array cost (Kendziorski et al., 2005).

2.5.2.3 Microarray data analysis

Once the variations mentioned above are well-managed, translating image information from a microarray experiment into biological meaning becomes the most important step for microarray analysis. A variety of statistical methods for low- and high-level analyses has been developed to meet this need (Pan, 2002; Chen, 2007).

2.5.2.3.1 Low-level analysis

The low-level analysis, or data preprocessing, includes image analysis (e.g., image segmentation and background correction), scaling/normalization, filtration and data transformation (Allison et al. (2002). In cDNA microarrays, background correction is done by subtracting the nonspecific signal from the spot signal. In contrast, Affymetrix data are proprocessed with the use of the perfect match and mismatch probes. Normalization in both cases makes different microarrays comparable. It involves standardizing microarray data to a certain intensity value. There are multiple options for choosing the value, such as the overall intensity of the microarray and the intensity of housekeeping genes. For negative normalization controls, target sequences acquired from a different organism are usually used. Microarray data are then log-transformed to improve the characteristics of the distribution of expression

intensities. The aim of the low-level analysis is to remove systematic variation across arrays and to allow comparisons between microarray experiments.

2.5.2.3.2 High-level analysis

High-level analysis is then applied to the data resulting from low-level analysis. This often is called data mining. This analysis mainly includes identification of the differentially expressed genes, classification analysis (or pattern identification), and interpretation of gene functions.

Fold change in expression was the first method used to evaluate whether genes are differentially expressed. However, it is considered to be an inadequate test statistic (Hsiao et al., 2004; Miller and Galecki, 2001), because it does not utilize variance to evaluate the associated level of confidence (Hsiao et al., 2004; Budhraja et al., 2003). In other words, using fold change only is not valid method for differential expression analysis.

The invalidness of fold change has led to the development of other methods that can minimize inferential errors – type I error (false positive error) and type II error (false negative error). The commonly used methods include *t*-test, ANOVA, logistic regression, and survival analysis. Because microarrays involve multiple testing, α-values should be adjusted. Bonferroni correction may be one of the options for the adjustment. However, because there are thousands of genes simultaneously tested in a microarray experiment, it appears impossible to identify differentially expressed genes that can reach the significance adjusted by Bonferroni correction. Therefore, less conservative correction methods are needed (Draghici, 2002). False discovery rate (FDR) is the approach that is commonly used in microarray analysis. It was proposed by Benjamini and Hochberg (1995). Subsequently, new methods, including mixture-model methods (Allison et al., 2002; Do et al., 2005; Pounds and Morris, 2003; Datta and Datta, 2005)

have been developed to improve the original one (Allison et al., 2002; Tsai et al., 2003; Hsueh et al., 2003; Van der Lann et al., 2004). In these methods, genes are treated as two types - one represents those genes that are differentially expressed, and the other(s) represents the genes that are not differentially expressed (Allison et al., 2002). These methods are much more powerful than the original one. They estimate a 'gene-specific' FDR, which is explained as the Bayesian probability of identifying a false differentially-expressed gene (Benjamini et al., 2001; Benjamini and Yekutieli, 2005; Dalmasso et al., 2005). Trying to maintain low FDRs often leads to low power to detect differential expression. Small sample sizes may make this situation worse. Obtaining a long list of differentially expressed genes, convincingly, is an initial success. However, this may confuse the investigators if these unorganised findings are not further interpreted. Analytic tools have been developed to meet the interpretive challenge posed by the long list of differentially expressed genes. Gene-class testing is one of the tools and has been widely accepted. Gene classes are usually based on gene ontology (GO) categories. There are several gene-class testing methods and software packages available (Osier et al., 2004; Khatri et al., 2002; Pavlidis et al., 2002, 2004). The principle behind most of the methods is based on statistic test to compare the number of 'significant' genes in a class with the number of genes expected under a particular null hypothesis. However, the null hypotheses are often poorly defined. In addition, how to handle multiple testing causes another problem with gene class testing analysis (Osier et al., 2004).

Classification analysis is an important part of data mining in a microarray experiment. This analysis aims to place objects into pre-existing categories (supervised classification) or a set of categories that previously are not available (unsupervised classification). Many classification algorithms have been developed for microarray data analysis. These algorithms divide genes or

samples into groups or clusters based on maximum similarity. They enable the identification of a gene signature or informative gene subsets. Clustering analysis is the frequently used method for unsupervised classification (Eisen et al., 1998). It can be divided into hierachical and nonhierarchical clustering. Most clustering analysis techniques are hierarchical. In hierachical clustering analysis, the number of nested classes is increased in the resulted classification. In comparison, nonhierarchical clustering analysis, such as *k*-means clustering (Varela et al., 2002), divides objects into different clusters without specifying the relationship between or among individual elements. Moreover, a self-organizing map is a neural-network-based divisive clustering approach (Tamayo et al., 1999). It assigns genes to a series of classifications based on the similarity of their expression vectors to reference vectors. Principal component analysis is also a powerful technique for analyzing gene expression data when combined with classification technique. This analysis requires the investigator to specify the number of clusters. In addition, linear discriminant analysis is an approach for supervised classification. In this approach, a training set that consists of all classes of interest is used to set up a model, then the model is used to classify an unknown sample and place it clearly into one of the established classes (Hakak et al., 2001).

Last but not least, microarray data need to be interpreted within the context of gene function and the functional relationships between genes. The development of a gene ontology database helps relate microarray data to existing biological knowledge.

2.5.2.4 Microarray-based identification of QTL for immune responses

Microarray technology has been used for identification of candidate genes involved in immune responses in model and farm animals. For example, Hutton et al. (2004) used the Incyte mouse GEM1 cDNA array to build a gene expression database from genes expressed in 65

diverse mouse tissues, including approximately 10% of immune tissues. As a result, 360 genes were identified as highly expressed only in mouse immune-specific tissues. The genes identified were candidate immune response genes in mice. Another example is the study conducted in chickens by Liu et al. (2001) for identification of candidate genes for Marek's disease resistance and susceptibility. In their results, 15 candidate immune response genes were identified.

2.5.3 In-silico genomics-based approaches

2.5.3.1 In-silico genomics

Just as its name implies, *in-silico* genomics is an interdisciplinary combination of computer science and genomics. It may overlap with comparative genomics because they both perform studies based on sequence similarity.

The prerequisites for conducting *in-silico* genomics studies are a variety of well-established databases and bioinformatics tools. Based on the resources, *in-silico* genomics functions at a variety of levels (genomic DNA, mRNA, protein). The strategy that is often used in *in-silico* genomics is a similarity search. The similarity stems either from the relative stability of intra-species inheritance or from inter-species evolutionary conservation (such as a homolog and other conserved elements). The Basic Local Alignment Search Tool (BLAST) is one of the bioinformatics tools that was developed for similarity search. The structure and function of a given DNA sequence could be predicted based on the similarity between the query sequence and subject sequence whose structure and function are known.

2.5.3.2 In-silico genomics-based identification of QTL for immune responses

With the increasing availability of sequences, expressed and genomic, a comparative genomics-based approach also provides an opportunity to identify immune response genes using bioinformatics tools. Despite the fact that this approach can not generate a new list of candidate

genes, it provides an easy and feasible approach to transferring information from species to species. For example, Smith et al. (2004) used an *in-silico* approach to identify potential chicken orthologues for human immune-related sequences. A total of 185 immune-related sequences were found, 95 of which may represent sequences of genes not previously present in GenBank/EMBL nucleotide and protein databases.

2.6 Molecular basis of antibody-mediated immunity

2.6.1 Genes associated with antibody-mediated immunity

To date, several causative genes that are involved in Ab production have been identified in animals, including the mouse. These genes can be roughly divided into four groups as follows. First, the genes controlling basal immunoglobulin level and/or Ab production, such as the genes *btk* (Scher, 1982; Tsukada et al., 1993; Vetrie et al., 1993), *gp39* (Aruffo et al., 1993; Kroczek et al., 1994; Notarangelo et al., 1992), *Rag* (Taccioli and Alt, 1995) and *ada* (Abbott et al., 1986; Gossage et al., 1993; Woloschak et al., 1987; Gossage et al., 1993). Second, MHC locus immune response genes. Third, *Igh* locus immune response genes. Last, genes at other loci, such as *IR-2* (Roopenian et al., 1993), *Thy1* (Young et al., 1976) and minor histocompatibility loci (Gasser et al., 1974). It has been observed that the effects of some of these genes can be strongly modulated by their interactions with background genes (generally standing for genes unlinked to MHC or *Igh* loci). Unfortunately, the background genes are mostly unknown.

2.6.2 Genetic markers associated with antibody-mediated immunity

Experimental data have revealed that immune response genes at MHC and Igh loci account for approximately 10% to 15% of the variation of Ab production, respectively. This suggests that non-MHC and non-*Igh* genes collectively should have a large influence on the

regulation of Ab production. It is possible to identify these genes with genetic markers. In fact, this identification has been made in both mice and chickens using genetic lines divergently selected for anti-SRBC response levels. In mice, Peul et al. (1995, 1996) conducted a genome-wide scan for genes or markers associated with anti-SRBC titers using Biozzi mice that express divergently selected anti-SRBC levels. Their results suggest that in addition to immunomodulatory genes over MHC and *Igh* regions, some unknown immunomodulatory genes were located near other markers, including *D4Mit31, D6Mit5, D8Mit57, D10Mit14* and *D18Mit19* over distinct chromosomal segments. Their contributions to anti-SRBC variation ranged from 3% to 13%. Similar studies also were conducted in chickens. Siwek et al. (2003) identified several markers associated with anti-SRBC titers on chicken chromosomes 3, 5, 6, 9, 10, 15, 16, 23 and 27 using F_2 birds produced by egg-laying chickens selected for high or low anti-SRBC levels. In addition, Yonash et al. (2001) reported that *ADL0146* on chromosome 2 was associated with variation of anti-SRBC level in segregating populations originated from crosses between broiler lines divergent for high and low Ab response to *E. coli*.

2.7 Summary of the literature review

The literature review discussed some features of the chicken that are related to its roles as a food animal in agriculture and a model animal for studying vertebrate immune response. Two types of resource populations for QTL identification including inbred lines and divergently selected lines also were compared in terms of advantages and shortcomings. Emphasis has been placed on the recent understanding of immune response, and on the technical problems and switches in genomics-based approaches for QTL identification.

The understanding of immune response was greatly deepened by many new findings. These findings were mostly concentrated on the mechanisms that underlie immune response, such as how immune response is activated, how a pathogen is recognized, how immune cells differentiate and mature after activation, and how major cellular and molecular components of immune response interact with each other. The understanding of the relations between natural disease resistance and immune response also was increased by identification of genes that underlie both traits. However, understanding of the molecular basis for the mechanisms that underlie immune response remains limited, although a few genes and genetic markers associated with immune response have been identified. Identification of new genes and genetic markers is essential for defining the molecular basis of immune response. Classic and new genomics-based analytic approaches, such as linkage analysis, LD analysis, genome-wide expression profiling, and *in-silico* genomics-based analysis have been developed to meet this challenging task. Use of divergently-selected lines may facilitate this process. Genetic lines divergently selected for anti-SRBC response have been developed, which provide a useful resource for identification of associated genes or genetic markers. These genes or genetic markers will contribute to the understanding of the molecular basis of immune response. In the following chapters of this dissertation, the identification of genes and genetic markers associated with anti-SRBC response will be described.

CHAPTER 3

Development and characterization of resource populations for QTL mapping

3.1 Abstract

Divergently selected lines and segregating crosses derived from them constitute a useful resource for the identification of quantitative trait loci (QTLs) for economically important traits. The objective of this project was to develop a resource population for the identification of genetic markers associated with antibody response to sheep red blood cells (anti-SRBC) in chickens. Birds from the 28th generation of lines divergently selected for high (H) or low (L) anti-SRBC titers were used as parental lines to develop reciprocal crosses, including F_1, F_2 and backcross (BC). As expected, the long-term selection for anti-SRBC caused significant phenotypic divergence (p<0.05) of the H and L lines, with the mean anti-SRBC titers of 11.5 for H and 2.6 for L. The data suggest that frequencies of alleles that underlie antibody response to SRBC have differentiated between the lines. Candidate marker analysis also supported this hypothesis. Heterosis for anti-SRBC response was not significant (p>0.05). The mean anti-SRBC titers of the 4 groups of F_2 progeny ranged from 6.3 to 7.5, while those of the 8 groups of BC progeny ranged from 3.9 to 13.3, suggesting that alleles that influence anti-SRBC titers were segregating in the resource population. In addition, phenotypic data from the resource population showed no significant sex, reciprocal, or maternal effects, making it appropriate for use in QTL analysis.

Keywords: antibody response, *Gallus gallus*, resource population, selection

3.2 Introduction

Divergently selected lines have been widely used for QTL analysis in animals, including swine (Kadarmideen and Janss, 2007), chicken (Yonash et al., 2001; Lagarrigue et al., 2006) and mouse (Puel et al., 1996; Horvat et al., 2000; Keightley et al., 1993). Selection leads to phenotypic divergence if a trait has moderate to high heritability. The increasing use of these lines for QTL analysis is probably because frequencies of alleles at most QTLs that underlie a selected trait differentiate due to selection. This results in the fixation or near-fixation of the favorable or unfavorable alleles (Andersson and Georges, 2004). Keightley et al. (1993) demonstrated that some QTL locations could be directly identified from marker allele frequency differences between divergently selected lines. Divergently selected lines also can be used to generate segregating populations, such as F_2 and backcross, for linkage-based QTL mapping or validation of candidate markers.

The level of antibody production is a quantitative trait with moderate heritability (Feingold et al., 1976; van der Zijpp et al., 1980; Pinard et al., 1992). Specific estimates of heritability range from 0.18 to 0.36. Response to divergent selection for anti-SRBC titers supports that anti-SRBC level is a polygenic trait (Siegel and Gross, 1980; van der Zijpp et at., 1980, 1983). The phenotypic divergence between the selected lines implies differentiation of allele frequencies at QTLs associated with anti-SRBC response in the two lines. It has been demonstrated that different MHC alleles, which are critical for antibody response, differentiated in the divergently selected anti-SRBC lines (Dunnington et al., 1984; van der Zijpp et al., 1987; Martin et al., 1990). The genetic architecture of anti-SRBC response, i.e., the heritability, heterosis, maternal and sex effects, in the resource population derived from the selected lines has been characterized (Boa-Amponsem et al., 1997). The effect of heterosis on anti-SRBC response

has not been consistent in the literature (Siegel et al., 1982; Ubosi et al., 1985a; Pinard and van der Zijpp, 1993; Boa-Amponsem et al., 1997).

Genetic lines divergently selected for high (H) or low (L) antibody titers in primary anti-SRBC response have been established for mouse (Feingold et al., 1976). These lines have been used for the identification of QTL associated with anti-SRBC titers (Puel et al., 1995, 1996). Similar lines also have been established in the chicken (Siegel and Gross, 1980; Van der Zijpp et al., 1983), although they have not been used for QTL analysis. Useful resource populations for QTL mapping consist of parental lines and crosses including F_1, F_2 and backcross populations. The objective of this study was to develop a resource population that could be a resource for mapping QTLs associated with anti-SRBC levels in the chicken. The utility of the resource population was initially examined by testing for the distribution of candidate markers associated with antibody titers, is described by Kaiser and Lamont (2002) and Kaiser et al. (2002).

3.3 Materials and Methods

3.3.1 Animals and crosses

The experimental lines originated from a random-bred White Leghorn foundation stock. The anti-SRBC levels of the lines and the divergent selection program have previously been described (Siegel and Gross, 1980). Briefly, birds between 41 and 51 days of age were selected for high or low antibody titers 5 days after a single intravenous injection of 0.1mL of a 0.25% suspension of SRBC. Anti-SRBC titers were determined by haemoagglutination assay, in which anti-serum that underwent 2-fold dilution multiple times was put into v-bottomed 96-well plate containing a 0.25% suspension of SRBC, and then this mixture was incubated at 37˚C for 4 hrs for antibody titer reading. Titers were expressed as $\log_2$ of the reciprocal of the highest dilution

in which there was hemagglutination. In this experiment, birds from the 28th generation of this two-way selection experiment and crosses derived from them were used. The mating scheme for the crosses is shown in Figure 3.1. In this scheme, F_1 progeny, HL and LH, were generated by reciprocal crosses of birds from the 28th generation of the selected lines, H(♀)×L(♂) and L(♀)×H(♂), respectively. The F_1 progeny were intercrossed {LH(♀)×LH(♂), HL(♀)×HL(♂),HL(♀)×LH (♂) and LH(♀)×HL(♂) } to generate 4 groups of F_2 progeny, giving rise to LHLH, HLHL, HLLH and LHHL stocks, respectively. The F_1 progeny also were reciprocally backcrossed to each of the parental lines {LH(♂)×L(♀), HL(♀)×L(♂), L(♀)×LH(♂), L(♀)×HL(♂), HL(♀)×H(♂), LH(♀)×H(♂), H(♀)×LH(♂) and H(♀)×HL(♂)} to generate 8 groups of backcross progeny, including LHLL, HLLL, LLLH, LLHL, HLHH, LHHH, HHLH and HHHL stocks, respectively.

3.3.2 Preliminary assessment of the usefulness of the resource population

Previously described microsatellite markers shown to segregate with antibody titers in other populations were tested for association with anti-SRBC in our resource population. Genomic DNA isolated from 48 birds per selected line (H and L) was used as a template in PCR as described by Smith et al. (2000). The markers, previously shown to be associated with antibody response to *Salmonella enterica* vaccine, include *ADL020, ADL138* and *ADL198* (Kaiser and Lamont, 2002; Kaiser et al., 2002). Primers previously described by Kaiser and coworkers (Kaiser and Lamont, 2002; Kaiser et al., 2002) were used for amplification, and the products were analyzed on an ABI377 automated sequencer (PE biosystems) using GeneScan 3.1 software (Applied Biosystems).

3.3.3 Statistical analysis

The GLM procedure of SAS was used to evaluate sex and line differences in anti-SRBC titers within and between the selected lines. Pair-wise differences among the mean anti-SRBC titers of the H, L, backcross (BC), F_1, and F_2 stocks were tested for significance using the Student's *t* test. Heterosis, reciprocal cross effect, maternal effect, and recombination effect also were assessed. Frequency of each microsatellite allele was calculated, and statistical significance of allele frequency differences between the parental lines was evaluated by the χ^2-test.

3.4 Results and Discussion

3.4.1 Phenotypes in the resource population

The mean anti-SRBC titers in the 28th generation produced by the birds used from the H and L lines were 11.5 and 2.6, respectively (Table 3.1). This result is consistent with previous reports (Boa-Amponsem et al., 1997), suggesting additive genetic variation for primary antibody response to SRBC at 5 days post-injection. This result also suggests that alleles associated with anti-SRBC response in these lines were differentiated. This differentiation is expected, as previous investigations showed that the L line reached a plateau in anti-SRBC response and did not overlap with the H line after 14th generations of selection (Martin et al., 1990). In addition, there was no difference in the mean anti-SRBC titers between sexes (data not shown), which is consistent with previous reports (Siegel and Gross, 1980; Boa-Amponsem et al., 1997). Thus, the mean anti-SRBC titers for each line shown in Table 3.1 is the average of the two sexes.

The mean anti-SRBC titers of F_1 progeny produced by reciprocal matings were 7.8 and 8.3 (Table 3.1, Figure 3.1). The mean anti-SRBC titers of F_2 and BC progeny were dependent on the types of crosses used for their generation, which classified F_2 and BC progeny into 4 and 8

groups, respectively. The mean anti-SRBC titers of the 4 groups of F_2 progeny ranged from 6.3 to 7.5, while those of the 8 groups of BC progeny ranged from 3.9 to 13.3 (Table 3.1, Figure 3.1). All crosses except HHHL backcross progeny fell within the range of the selected lines. The rank of each cross for the mean anti-SRBC titers varied with the amount of genetic elements inherited from the respective selected lines. For example, the more inheritance from the H line, the higher the mean anti-SRBC titers of the cross. This result suggested that alleles influencing anti-SRBC titers that segregate in the F_2 and BC progeny would be appropriate for validation of candidate markers associated with the trait.

The F_1 birds had higher, but not significantly different mean anti-SRBC titers from the parental average ($p>0.05$), suggesting lack of heterosis for anti-SRBC titers. This is consistent with some (Pinard and van der Zijpp, 1993) but not all previous studies (Siegel et al., 1982; Boa-Amponsem et al., 1997). In addition, the reciprocal cross effect (HL-LH) and the maternal effects (LL F_1- F_1LL or HH F_1-F_1HH) were not significant ($p>0.05$).

3.4.2 Informativeness of the resource population

The three candidate microsatellite markers, including *ADL020*, *ADL138* and *ADL198*, exhibited significantly different allele frequencies between the selected lines ($p<0.001$), with some alleles being line-specific (Table 3.2). For example, a 94 bp allele at *ADL020* was detected only in the L line while a 106 bp allele was observed only in the H line. This observation is consistent with earlier reports of segregation of MHC alleles with anti-SRBC response (Dunnington et al., 1984; van der Zijpp et al., 1987; Martin et al., 1990). Moreover, because at least one marker, *ADL198*, is not located near either the MHC or the *Igh* loci, the selected lines could prove useful for identification of non-MHC/*Igh* genes that significantly influence anti-SRBC titers. Because candidate markers were previously shown to be associated with antibody

response to *Salmonella enterica* vaccine (Kaiser and Lamont, 2002; Kaiser et al., 2002), genes influencing antibody production in response to SRBC may be similar to those for anti-*Salmonella enterica* antibody production. This hypothesis is supported by observations that chicken lines with elevated antibody titers to one antigen had high antibody responses to other antigens (Heller et al., 1992; Yunis et al., 2002). Genetic markers like these prove useful in marker-assisted selection for improving general disease resistance in animals.

Table 3.1 Means and standard devations of antibody response by chickens in the high (H)-, low (L)-, backcross (BC)-, and F_2-resource populations five days post-injection of sheep red blood cells (SRBCs)

Population/*n*[a]	Classification[b]	Mean ±SD[c]
Selected/138	L	2.6±1.11[d]
Selected/120	H	11.5±2.83[f]
F_1/70	LH	7.8±0.96[e]
F_1/65	HL	8.3±1.51[e]
BC_2/57	LHLL	3.9±1.51[d]
BC_2/44	HLLL	4.6±1.68[de]
BC_2/53	LLLH	4.8±1.27[de]
BC_2/48	LLHL	5.9±2.06[e]
BC_1/43	HLHH	10.4±2.45[f]
BC_1/59	LHHH	10.5±3.33[f]
BC_1/59	HHLH	10.5±3.59[f]
BC_1/24	HHHL	13.3±4.43[f]
F_2/39	LHLH	6.3±2.44[e]
F_2/38	HLHL	6.4±2.12[e]
F_2/40	LHHL	7.1±1.85[e]
F_2/42	HLLH	7.5±2.23[e]

[a] *n* is the number of birds from which antibody response to SRBC was determined.

[b] H and L represent the parental lines divergently selected for high (H) and low (L) anti-SRBC titers, respectively. HL and LH in the F_1 population represent birds produced by H(♀)×L(♂) and L(♀)×H(♂) crosses, respectively. LHLL, HLLL, LLLH, LLHL, HLHH, LHHH, HHLH and HHHL in BC population represent birds produced by LH(♀)×L(♂), HL(♀)×L(♂), L(♀)×LH(♂), L(♀)×HL(♂), HL(♀)×H(♂), LH(♀)×H(♂), H(♀)×LH(♂) and H(♀)×HL(♂) crosses, respectively. LHLH, HLHL, LHHL and HLLH in the F_2 population represent birds produced by LH(♀)×LH(♂), HL(♀)×HL(♂), LH(♀)×HL(♂) and HL(♀)×LH (♂) crosses, respectively.

[c] Values within the column of antibody titers (log_2 transformed) with no common superscript differ significantly ($p<0.05$).

Table 3.2 Allelic frequencies of candidate microsatellite markers that were previously identified as associated with immune response

Marker	*ADL020*	*ADL138*	*ADL198*
Allele (bp)[a]	94, 98, 106[d]	113, 119	115, 117, 119, 123
H/*n*=48[b]	0.00, 0.61, 0.39[e]	0.56, 0.44	0.97, 0.03, 0.00, 0.00
L/*n*=48[c]	0.13, 0.87, 0.00	1, 0.00	0.24, 0.05, 0.66, 0.05
χ^2/*p*-value	53.4/<0.001	53.8/<0.001	110.7/<0.001

[a] Alleles at each candidate microsatellite marker locus indicated by the size of the allele in base-pairs (bp). The candidate markers were associated with antibody titers to *S. enterica*, which was described by Kaiser and coworkers (2002).

[b,c] Allele frequencies estimated from 48 individuals in each selected line. H and L represent the chicken lines divergently selected for high or low antibody titers to sheep red blood cells, respectively.

[d,e] The sizes of the alleles at each candidate marker and their corresponding frequencies in the selected lines.

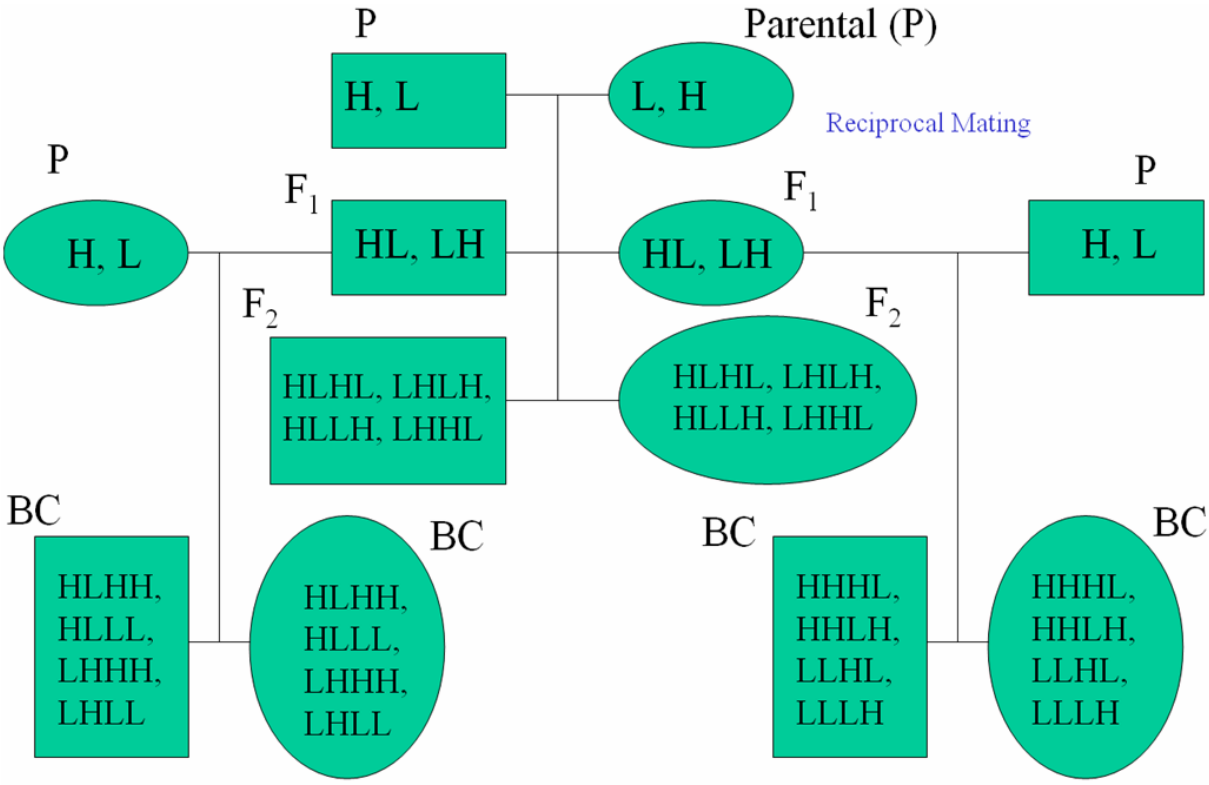


Figure 3.1 Resource population development scheme. The diagram above illustrates the mating scheme for the development of crosses. The crosses including F_1, F_2 and backcross (BC) were developed from the parental lines (P). Each cross has reciprocal matings. The rectangles and the ovals represent male and female bird, respectively. H and L represent the lines divergently selected for high or low antibody titers to sheep red blood cells, respectively, or represent genetic elements inherited from each of the lines.

CHAPTER 4

Identification of candidate DNA markers associated with primary antibody response to sheep red blood cells in chicken

4.1 Abstract

Randomly amplified polymorphic DNA analysis provides a technically easy, rapid, and inexpensive method for conducting a genome-wide scan to identify markers associated with QTLs using divergently selected lines. Here, I describe DNA markers associated with levels of antibody titers produced in response to a challenge by sheep red blood cells (anti-SRBC). Five hundred and fifty five random primers were used in the preliminary screening of DNA pools established from chickens divergent for level of antibody titers. The resulted informative random primers were further evaluated on 48 individual DNA samples from each of the divergent anti-SRBC lines. Six of them amplified line-specific RAPD fragments. Seven SCAR markers were converted from the line-specific fragments amplified by 4 of the random primers. These SCAR markers were on chicken chromosomes 1 (2 markers), 2 (2), 4 (1), 8 (1), and 13 (1), which was determined by genetic and physical mappings. Fifty-two SNPs were detected in the SCAR markers and their flanking regions. The subsequent SNP-QTL analysis indicated that the allelic frequencies of the SCAR markers were significantly different ($p<0.0025$) between the parental lines, although only two of them were confirmed in the segregating population ($p<0.05$). The candidate markers will be useful in efforts to understand the genetic control of humoral immune response and to develop breeding programs of marker-assisted selection for antibody production in chickens.

Keywords: DNA markers, *Gallus gallus*, humoral immunity, SNP (single nucleotide polymorphism)

4.2 Introduction

Public health concerns about the use of antibiotics in livestock and poultry feed have increased interest in raising animals with strong natural immunity (The New York Times, February 10, 2002). One approach for identifying and raising animals with strong natural capacity to resist diverse pathogens is by the use of genetic markers. Increased disease resistance may allow reduced use of antibiotics in animal feed. Studies have shown that genetic factors associated with humoral immune response including those related to antibody production are involved in animal's disease resistance (Gross *et al.,* 1980; Plant & Glynn, 1982; Siqueira *et al.,* 1985; Martin *et al.,* 1986; Stiffel *et al.,* 1987; Gross *et al.,* 2002; Yunis *et al.,* 2002). However, identifying the genetic factors is challenging in vertebrates because the level of humoral immune response or antibody production is a quantitative trait controlled by multiple genes (Feingold *et al.,* 1976; Biozzi *et al.,* 1979; Ibanez *et al.,* 1988). As a result, knowledge about the molecular basis of the trait remains limited.

Experimental populations in both the mouse (Biozzi *et al.,* 1972; Feingold *et al.,* 1976) and chicken (Siegel & Gross, 1980) that showed divergence in antibody levels in response to sheep red blood cells (anti-SRBC) have been used to identify genetic markers associated with genes that influence antibody production. In the mouse, selected lines divergent for antibody titers were used by Puel et al. (1995, 1996, 1998) to identify and characterize genetic markers for quantitative trait loci (QTLs) associated with anti-SRBC. The reciprocal backcrosses of the mouse lines also were used to confirm the association of *Igh* gene and the major histocompatibility complex (MHC) with anti-SRBC response. The non-MHC and *Igh* genes, however, accounted for about 40% of phenotypic variation observed in antibody levels in the segregating population.

As in the mouse, DNA markers associated with antibody response have previously been reported for the chicken (Yonash *et al.,* 2001; Siwek *et al.,* 2003; Zhou *et al.,* 2003). In laying hens, Siwek et al. (2003) used an F_2 population developed from a cross between chickens divergent for high or low anti-SRBC titers to detect a total of six QTLs associated with anti-SRBC titers on different chicken chromosomes. Other chicken populations also have been used in the search for QTLs associated with primary anti-SRBC, including meat-type chickens (Yonash *et al.,* 2001; Zhou *et al.,* 2003). For example, Yonash et al. identified a QTL on chromosome 2 using broiler lines divergently selected for antibody production to *E. Coli* vaccine (Yonash *et al.,* 2001). However, this QTL was different from that identified by Siwek et al. (2003). This difference in identification of candidate markers may be due partially to the use of different population, which have their own characteristics, such as different breeds and the subjects of different selection programs. These results collectively suggest that more work will be needed for identification of candidate DNA markers or QTLs responsible for antibody production.

4.3 Materials and Methods

4.3.1 Animals, DNA samples, and template preparation

The resource population consisting of the divergently selected lines and the crosses derived from these was used, which has been described in Chapter 3. In the preliminary analysis, 20 birds from each of the selected lines (H and L) were used to create two DNA pools according to Smith et al. (2002). The pools and 2 individual DNA samples from each of the selected lines then were used to screen for line-specific DNA fragments. Primers revealing line-specific fragments were further tested on 48 birds from each of the H and L lines. Thirty-two birds from

each of the backcross 1 and 2 (BC_1 and BC_2) populations generated from matings of F_1s to H and L line birds, respectively, also were used to test primers that revealed line-specific fragments.

4.3.2 Randomly amplified polymorphic DNA (RAPD) analysis

Blood was collected from each bird into tubes containing 0.5M EDTA by brachial venipuncture. Genomic DNA was isolated from 50 ul aliquots of each sample using a standard salt-precipitation procedure (Bartlett *et al.,* 1996). Genomic DNA samples were dried down and dissolved in sterile water to a concentration of 50ng/ul.

A total of 555 10-base random primers (Operon Technologies) were used in the initial screening of DNA pools from the selected lines to identify informative primers that reveal H or L line-specific DNA fragments. Each amplification reaction was in a final volume of 10uL containing: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 75 ng template DNA, 40 ng primer, 200 uM of each dNTP and 1 unit of Amplitaq DNA polymerase (Applied Biosystems Inc.). The PCR was carried out in a PTC100 (MJ Research) thermal cycler using the following cycling conditions: initial denaturation at 95°C for 5 minutes, followed by 34 cycles of denaturation at 94°C for 1 minute, annealing at 35°C for 45 seconds, and extension at 72°C for 2 minutes, then a final extension at 72°C for 5 minutes.

4.3.3 SNP analysis

The RAPD fragments that were line-specific on both DNA pools and individual samples were gel-purified using the Qiaquick Gel Extraction kit (Qiagen Ltd.), cloned in the TA cloning kit (Invitrogen), and sequenced using the BigDye cycle sequencing kit as previously described (Smith *et al.,* 2000). One kilo-base (kb) length of sequences that are 5 kb and 10 kb up- or down-stream of the sequenced DNA fragments were also retrieved from chicken genome sequence through Chicken Genome Browser Gateway on the webpage:

http://genome.ucsc.edu/cgi-bin/hgGateway. Primers were designed from each sequence for PCR using PRIMER 3 (Rozen & Skaletsky, 2000). Amplicons produced using each pair of primers were scanned for SNPs by sequencing with the use of bioinformatics tools Phred, Phrap and Consed (Gordon *et al.,* 1998). Genotyping of the backcross panel for informative SNPs within each sequence was done by re-sequencing as previously described (Nickerson *et al.,* 1998; Smith *et al.,* 2000).

For the genetic mapping, the parental samples of the East Lansing chicken genetic mapping reference panel (Crittenden *et al.,* 1993) were used to evaluate the informativeness of the SNPs identified within each RAPD amplicon designated as a putative DNA marker. Markers with informative SNPs, or those for which the sire and dam had different genotypes, were mapped by multipoint linkage analysis (Cheng *et al.,* 1995). For the radiation hybridization (RH) mapping, PCR was carried out as suggested by Morisson et al. (2002) using the same primers and conditions used for the sequence-based genetic mapping.

4.3.4 Bioinformatics analysis

The sequences of the RAPD fragments were used to determine their chromosomal locations based on sequence alignment against the *Gallus gallus* genome sequence, which is available at: http://genome.ucsc.edu/cgi-bin/hgBlat?command=start. The candidate genes at or near these sequences, which have known or putative functions related to immune response, were searched for using the Map Viewer tool available on the webpage of the National Center for Biotechnology Information.

4.3.5 Statistical analysis

The statistical significance of allele frequency difference at each candidate DNA marker locus between the selected lines was evaluated by the χ^2-test. The association of each marker

with anti-SRBC titers in the backcross population was tested using the GLM procedure of SAS. In the GLM analysis, the main effects mating type (BC_1 and BC_2) and each SNP were tested without consideration of any interaction terms. The effect of sex was not included because it was not significant ($p>0.05$).

Haploview3.32 (http://www.broad.mit.edu/mpg/haploview/) was used to test Hardy-Weinberg Equilibrium of each SNP, calculate pairwise LD between SNPs, construct haplotypes, and perform case-control association studies for both single markers and haplotypes using pedigree information from the BC_1 and BC_2 populations. A total of 64 individuals from the backcrosses was used in the analysis. These individuals were grouped into 26 families based on parental origin. Case-control association studies were performed separately for each of the two backcross populations by converting anti-SRBC titers into two dummy values, '1' for 'Control' and '2' for 'Case'. Individuals with anti-SRBC titers >9 in BC_1 or <6 in BC_2 were labeled as 'Case', while the rest were labeled as 'Control'. Here, the cutting-offs were set to make the numbers of 'Case' and 'Control' roughly equal. Hardy-Weinberg equilibrium was tested for each of the single SNP markers and halpotypes before case-control study was performed. The significance of association between anti-SRBC titers and single marker/haplotype was estimated by Chi-square test. A permutation test also was performed with 1000 permutations to obtain a measure of significance corrected for multiple testing biases.

4.4 Results

4.4.1 Sequence analysis and mapping

About 6 RAPD fragments on average were amplified by each of 555 random primers (data not shown). Out of the 555 random primers tested, six amplified line-specific fragments in

the pooled and individual DNA samples (Table 4.1, Figure 4.1). The estimated sizes of the line-specific fragments that were amplified by the 6 primers ranged from 500 bp to 1200 bp. Frequencies of the line-specific fragments ranged from 51-96%. Seven sequence characterized amplified region (SCAR) markers were converted from the line-specific RAPD fragments amplified by 4 random primers because they had higher frequencies (Table 4.2). Each of the SCAR marker sequences was submitted to GenBank, and was assigned an accession number (Table 4.2).

Within the SCARs and their flanking regions, 52 SNPs were identified using the selected lines (H and L) and the East Lasing (EL) genetic mapping reference panel (Table 4.3, Figure 4.2). The 6 SNPs detected in the East Lansing population were used to map the SCARs by linkage analysis. Two SCARs, *VTC02* (or *VPI0004*) and *VTC06* (or *VPI0005*), were assigned to the consensus linkage groups GGA4 and GGA8 with LOD scores of 11.1 and 13.2, respectively (Table 4.5, Figure 4.4, Figure 4.5). However, *VTC03* was unlinked (Table 4.5), and the other putative markers did not have an informative polymorphism in the East Lansing reference mapping panel. Sequence alignment of the 7 SCARs against the chicken genome sequence deposited in GenBank showed similarities to chicken chromosomes 1, 2, 4, 8, and 13 (Table 4.2).

In radiation hybridization mapping (Figure 4.4), *VTC03* was linked to *MCW153, LEI248, MCW087, MCW027* and *MCW009* on chromosome 2 with LOD>=6, which was consistent with the result from sequence alignment of the marker. However, *VTC02* only was linked to *MCW0131* on chromosome 2 with a LOD>=6, which was not consistent with its genetic mapping data (GGA4). Other markers also were not mapped. These cases occurred probably because of the low-resolution chicken radiation hybridization map at that time (Table 4.6, Figure 4.6).

4.4.2 SNP analysis

Allele frequencies between H and L birds for some of the SNPs identified in the SCAR markers were significantly different (p<0.0025) (Table 4.3, Figure 4.3). Three markers, including *VTC02, VTC04* and *VTC06*, also were significantly (p<0.05) associated with anti-SRBC response in the backcross population based on the GLM analysis (Table 4.3).

The pairwise LDs between the SNPs with LOD scores are shown in Table 4.7. Few SNPs were not in Hardy-Weinberg Equilibrium (data not shown). Based on the pairwise LDs between SNPs, LDs were plotted and haplotypes with frequencies were constructed in the backcross populations (Figure 4.7). The case-control association studies indicated that *VTC01* and *VTC03* in BC_2 and *VTC04* and *VTC06* in BC_1 were significantly associated with anti-SRBC titers (p<0.05) (Table 4.8, Figure 4.8), which was partially consistent with the GLM analysis.

The differences in anti-SRBC titers among genotypes of selected SNP for each of the candidate markers indicated that the alleles G at *VTC02S5*, G at *VTC03S1*, C at *VTC04S1*, C at *VTC05S1* and T at *VTC07S1* were significantly associated with QTL alleles that have negative effects on anti-SRBC titers, while the alternative alleles had increasing effects (p<0.01) (Table 4.4).

4.4.3 Bioinformatics analysis

Bioinformatics analysis indicated that there were 18 immune response-related genes located near (<2.5 Mb) the SCAR markers (Table 4.9). These genes include those close to *VTC01* (*TCF7, LTC4S* and *CD74*), *VTC02* (*C1QTNF7, LOC428465* and *CYTL1*), *VTC03* (*LOC420912, cadherin* and *CDH10*), *VTC04* (*LOC428465, LOC428464, RCJMB04_3g3, LOC420756* and *LOC420755*), *VTC05* (*BTG1* and *SOCS2*), *VTC06* (*BCL10*), and *VTC07* (*CD99*). The distances from their corresponding SCAR markers ranged from 111 kb to 2,250 kb.

4.5 Discussion

In this study, seven line-specific DNA fragments were identified in the selected lines through an approach that combines LD-based analysis with RAPD-based genome-wide scan (Table 4.2, Figure 4.1). The principle behind this is that a QTL associated with the selected trait could be identified based on allele frequency differences of QTL and DNA markers that are in LD with the QTL among divergently selected lines (Keightley et al., 1993). In divergently selected lines, the favorable alleles of QTLs and their flanking regions under strong selection could be fixed or near-fixed (Andersson and Georges, 2004; Siegel et al., 2006). In this study, the divergently selected lines used were developed from 28 generations of selection for high or low anti-SRBC titers. As indicated in Chapter 3, most of QTLs associated with anti-SRBC response were differentially accumulated in the lines.

Due to randomness of primer sequences used in a RAPD-PCR reaction, the RAPD technique provides an effective approach to genome-wide screening for differentially-accumulated alleles at QTLs or neighboring marker loci. In this study, 555 random 10-mer oligo-nucleotide primers were used to screen for informative markers. This amount of random primers provides a theoretically estimated coverage of 40 Mb of genome sequence, which accounts for about 3.3% of the whole chicken genome (about 1.2×10^9), assuming that the number of detectable amplicons generated for each primer is about 6 on average, as indicated by the present work. This means the chicken genome was scanned for candidate QTLs/markers at a ratio of one examined base to 30 unexamined bases.

Bioinformatics analysis of these 7 line-specific fragments indicated that they are located near immune response related-genes (Table 4.9). More importantly, several genes including

MHC class II (*CD74*), complement component (*C1QTNF7*), and T-cell receptor (*LOC428464, LOC428465*) are known to play roles in antibody response (Malnati et al., 1992; Pieters, 2000; Mukhopadhyay et al., 2004; Reid and Porter, 1981; Fearon and Carroll, 2000). This list of genes provided a resource to identify causative genes that underlie antibody response. However, this study did not identify an *Igh* gene that can influence anti-SRBC titers (Puel et al., 1996). This failure to discover QTLs with major effect is probably because the number of primers used was limited compared to the size of chicken genome. The number of QTLs identified by RAPD technique should be increased with the amount of random primers used in the RAPD-based genome scan.

Although a selective sweep could cause allele frequency differences at a QTL and its neighboring marker loci, other factors, such as small population size and genetic drift, could also contribute to it. To exclude this possibility from this study, the backcross population was used as a segregating population to validate the 7 informative markers derived from the line-specific fragments through linkage analysis. The principle behind this is that if a marker is truly linked to a QTL, the association between the marker and the trait affected by the QTL will still exist in linkage analysis. In contrast, if a marker is not linked to a QTL and the association between the marker and the trait affected by the QTL is due to genetic drift, the association will disappear in linkage analysis. Two statistical methods, the GLM procedure of SAS and case-control association analysis, were employed, because the former is based on analysis of variance, while the latter is based on the Chi square test. They may provide different statistical power in different experimental designs. In this study, 5 of the informative markers were validated by at least one of the two methods. *VTC04* and *VTC06* were validated by both of the methods. Moreover, it is interesting to note that *VTC01* and *VTC03* were validated only in BC_2 while *VTC04* and *VTC06*

were only validated in BC_1 by case-control association analysis. This phenomenon may be because the genetic makeup of different populations may influence QTL expression and detection power. This was supported by the observation that association tests were confounded by population stratification (Risch and Merikangas, 1997). In addition, haplotypes constructed in this study were used to test their associations with anti-SRBC titers in addition to single markers. It has been shown that using haplotypes in association studies may improve statistical power (Zhang et al., 2002).

The candidate markers identified in this study were all different from those previously reported by other investigators (Yonash *et al.,* 2001; Siwek *et al.,* 2003; Zhou *et al.,* 2003). One interpretation for the uniqueness of our candidate markers is that the resource population used in this study was different from those used in other studies. Studies of QTL identification in other animal species indicated that the characteristics of resource population, such as breed and mating scheme, could affect the power of QTL detection (Rupp & Boichard, 2003; Schulman *et al.,* 2004). Some QTLs can be detected easily in one population but not in another population. Another interpretation is that the approach for QTL identification in this study was different from those in other studies.

Different from previous studies using linkage analysis-based approaches, this study used an approach that combined the advantages of LD-based analysis with RAPD technique. Compared to linkage-based analysis, LD-based analysis was more efficient in QTL identification. For example, LD-based analysis could identify QTLs with small effects, finely map a QTL, and increase the power of QTL detection at a given sample size (Risch and Merikangas, 1996). This is because LD-based analysis takes advantage of historical recombination in a population. In contrast, linkage-based analysis can only identify QTLs with moderate to major effect, and its

mapping efficiency is readily affected by genetic and environmental noise (Andersson and Georges, 2004). For example, Crawford et al. (2006) discovered only a single QTL associated with nematode parasite resistance in sheep despite the moderate heritability of this trait and large scale of this study. Apparently, most of the genes that control the trait have relatively small effect. Moreover, Risch and Merikangas (1996) and Mathew (2001) found that results from linkage-based analysis were difficult to replicate. In addition, linkage-based analysis is an insensitive QTL mapping tool because it can only map a QTL to a large chromosomal region in which tens of genes may reside (Darvasi et al., 1993). Therefore, this study may be more efficient with more confidence in QTL identification than previous studies.

Table 4.1 Size and frequency distributions of selected RAPD fragments in the parental lines.

Primer[a]	Sequence[b]	Size (bp)[c]	Frequency[d]	
			H	L
Hokie1	5'-TGCGCCCTTC-3'	800	0	76
Hokie2	5'-GATGACCGCC-3'	1200	96	0
Hokie3	5'-CTCACGTTGG-3'	500	0	87
Hokie4	5'-ACATCGCCCA-3'	560, 580	81, 0	0, 82
Hokie5	5'-CTGATACGCC-3'	1050	51	0
Hokie6	5'-CAGACAAGCC-3'	800	0	68

[a] The name of the random primer used.

[b] The sequence of the random primer used.

[c] The estimated size of the selected RAPD fragment.

[d] % of birds in the parental line with the selected RAPD fragment.

Table 4.2 Sequence characterized amplified region (SCAR) candidate DNA markers for anti-SRBC response.

Marker[a]/primer[b]	Size[c] (bp)	Accession Number[d]	Chromosome:Coordinates[e]
VTC01/Hokie1	868	**AY435424**	Chr13:15,192,367-15,193,230
VTC02/Hokie2	1148	**AY435425**	Chr4:79,574,744-79,575,886[f]
VTC03/Hokie3	511	**AY435426**	Chr2:74,967,060-74,967,568
VTC04/Hokie4	600	**AY435427**	Chr2:50,346,631-50,347,236
VTC05/Hokie4	581	**AY435428**	Chr1:46,425,852-46,426,431
VTC06/Hokie4	489	**AY435429**	Chr8:19,189,926-19,190,404[f]
VTC07/Hokie4	528	**AY435430**	Chr1:131,019,193-131,019,716

[a] Locus identification for the SCAR marker that was developed from the line-specific RAPD fragment.

[b] Sequence of the random primer used to develop the SCAR markers.

[c] The size of each SCAR marker in base pair (bp).

[d] GenBank accession number for the consensus DNA sequence of the SCAR marker.

[e] Chromosomal coordinates of the markers within the genomic DNA sequence (http://genome.ucsc.edu/cgi-bin/hgGateway).

[f] *VTC02* and *VTC06* were also mapped by linkage analysis to *MCW0098* and *VTG2* with LOD scores of 11.1 and 13.2, respectively, using the East Lansing reference panel (Cheng et al., 1995).

Table 4.3 Single nucleotide polymorphism (SNPs) within and around sequence characterized amplified regions (SCARs) and the associations of the SNPs with anti-SRBC in the resource populations.

SNP ID[a]	Context[b]	Population[c]	Allelic frequency[d]			backcorss
			H/*n*	L/*n*	χ2/*p*-value	*p*-value/*n*[e]
VTC01u10S1	attgtttgg(C/T)gctgaagttga	H and L				
VTC01u10S2	taccagtcccta(A/G)tact	H and L				
VTC01S1	caatt(**C**/T)acctggaga	H and L	.013/40	.203/32	14.7/<.001	.098/61
VTC01S2	agacactc(C/T)tgactgtgtaccc	H and L				
VTC01S3	tctcttg(C/T)ccattt	H and L				
VTC01S4	ccatttgtagg(C/T)aaagctggaa	H and L				
VTC01S5	cccttaggaaac(C/G)acagtattt	H and L				
VTC01S6	gctcctttctc(G/T)gcaaca	H and L				
VTC01d5S1	tgtggaaaca(A/G)cacacagaa	H and L				
VTC01d5S2	aggggtgagg(C/T)tctgctgcc	H and L				
VTC02u10S1	ggctgagagctg(A/G)ttttct	H and L				.008/61
VTC02u10S2	ccc(A/G)taaccaaaactaaac	H and L				
VTC02u10S3	ggtggtttggc(A/G)tgatttata	H and L				.048/61
VTC02S1	tgtttct(C/T)cttgctg	H and L/EL				
VTC02S2	gacac(TG/CA)attttc	H and L				
VTC02S3	ctcctg(A/T)gggctg	H and L				
VTC02S5	cacaa(**A**/G)ccatg	H and L	.708/48	.000/48	105/<.001	.013/61
VTC02S6	aagtt(G/T)aaatac	H and L				
VTC02S7	tttaatga(A/C)ttcagaa	H and L				
VTC02S8	actta(G/T)tagtcaa	H and L				.014/61
VTC02S9	gcttg(A/G)ggctgt	H and L				
VTC02S10	gcaaa(C/T)cactttt	H and L				
VTC02S11	tcacc(A/G)gctttcc	H and L				
VTC02S12	tgttc(C/T)gtggct	EL				

Continued

SNP ID[a]	Context[b]	Population[c]	Allelic frequency[d]			backcross
			H/*n*	L/*n*	χ2/*p*-value	*p*-value/*n*[e]
VTC02S13	tgcag(C/T)gaagct	EL				
VTC02d10S1	gaccaaata(C/T)gtaagttgta	H and L				
VTC03u10S1	ctgaattatca(A/G)cttaccat	H and L				
VTC03S1	tgctgt(A/G)tgtac	H and L	.990/48	.340/47	90.3/<.001	
VTC03S2	aatagtg(A/T)aatcaga	H and L				
VTC03S3	atac(A/G)cagagatt	EL				
VTC03S4	gcatc(C/G)tgagctt	EL				
VTC03d5S1	tggtggtaa(C/G)atactcatca	H and L				
VTC04u10S1	aagcaccc(A/T)atcttagtct	H and L				
VTC04u10S2	gcttacaaga(A/G)aatattaa	H and L				.020/62
VTC04u10S3	tcttgctctt(C/T)aatgtataata	H and L				.002/62
VTC04u10S4	gtgtttcaata(A/T)aagaaatt	H and L				.002/62
VTC04u10S5	ctgcatattc(A/T)aatctgac	H and L				
VTC04S1	ttgta(**C**/T)cttatt	H and L	.000/48	.833/45	134/<.001	
VTC04S2	tgaggg(A/G)catgatg	H and L				
VTC04d10S1[f]	catcttcatg(C/T)caaagacctgc	H and L				.041/62
VTC05u5S1/S2	ttattaaaaaaaa(A/T)a(A/T)a	H and L				
VTC05S1	gagactt(**CT**/GC)acttcaa	H and L	.777/47	1.00/48	24.1/<.001	.067/58
VTC05d10S1	aaaggagattct(C/T)ttagctg	H and L				
VTC06u10S1	cttggc(A/G)aa(A/G)aaacaa	H and L				.083/49
VTC06u10S2	gaaccatc(C/T)ttacactactcaa	H and L				
VTC06S1	aca(A/G)acacagctt	H and L				
VTC06S2	aactgga(**C**/T)gtaaaaaac	H and L	.750/48	.533/46	9.68/<.025	.060/49
VTC06S3	aaaa(C/T)cttgagtatctcc	EL				
VTC06d5S1	aaaggaaatatc(A/T)tttctttt	H and L				.005/61
VTC06d5S2	aatgactcct(G/T)agttaagatt	H and L				.005/61
VTC07S1	agagtatccat(**C**/T)aagagctg	H and L	1.00/48	.787/47	22.8/<.001	

[a] SNP name was based on the following rule: the first five letters/digits are used for the identity of each SCAR marker described in this study, the six letter and the following digit(s) mean SNPs were identified in the flanking sequences that are 5 or 10kb up (u)- or down (d)- stream of the markers, and the letter 's' and the following digit(s) represent SNP name.

[b] Sequence context in which SNP was detected. Each SNP is highlighted by upper case letters.

[c] Populations in which SNPs were detected. H and L represented the lines divergently selected for high and low anti-SRBC, respectively (see Chapter 3); EL represents East Lansing genetic mapping reference panel.

[d] The frequency is that of the allele in bold for each SCAR with significance between the selected lines, H and L, indicated by Chi-square test statistic and *p*-value, correspondingly. *n*, the number of birds genotyped for the SNP tested.

[e] *p*-values indicate the statistical significance of association of each SCAR with anti-SRBC titers in backcross population (see Chapter 3); only the SNPs with *p*-value of less than 0.10 are listed. *n*, the number of birds used in the analysis.

[f] An insertion/deletion polymorphism, which was assigned as *VTC04d10P1*, was detected nearby the SNP.

Table 4.4 Differences in anti-SRBC titers between genotypes of selected SNPs for each candidate marker in the backcross population

SNP ID[a]	Genotype(n[b])	Mean±SD[c]	*F*-value/*p*-value
VTC01S1	CC(0)	-	0.89/0.3484
	CT(20)	8.94±3	
	TT(41)	8±3.54	
VTC02S5	AA(6)	12.5±2.35	12.90/<0.0001
	AG(19)	9.47±3.13	
	GG(36)	6.78±2.82	
VTC03S1	AA(16)	11±3.1	11.41/<0.0001
	AG(32)	7.75±2.94	
	GG(14)	6.07±2.67	
VTC04S1	CC(21)	5.9±1.87	15.77/<0.0001
	CT(24)	8.25±3.31	
	TT(17)	11±2.89	
VTC05S1	CC(51)	7.8±3.31	11.41/0.0013
	CG(7)	12.1±1.86	
	GG(0)	-	
VTC06S2	CC(17)	8.82±3.59	1.09/0.3459
	CT(28)	7.61±3.4	
	TT(4)	6.5±1.0	
VTC07S1	CC(40)	9.13±3.35	5.47/0.0067
	CT(17)	6.47±2.87	
	TT(3)	5.33±.577	

[a] SNP identities are as in Table 4.3.

[b] The number of birds with the genotype indicated before it.

[c] The mean and standard deviation of anti-SRBC titers for each genotype.

Table 4.5 Genotypes of the candidate markers in the East Lansing reference mapping panel

East Lansing mapping panel		VTC02	VTC03			VTC06
Bird ID#	Bird Type	SNP1	SNP1	SNP2	SNP3	SNP1
10394	sire	T	A/T	A/G	C/G	T
24000	sire	C/T	A/T	A/G	C/G	C/T
31821	dam	C	A	A	C	C
31824	dam	C	A	A	C	C
31830	dam	C	A	A	C	C
31838	dam	C	A	A	C	C
521A1	progeny	C/T	A	A	C	C/T
520B1	progeny	C/T	A	A	C	C/T
526C1	progeny	C	A/T	A/G	C/G	C/T
520D1	progeny	C	A	A	C	C
520E1	progeny	C	A/T	A/G	C/G	C/T
520F1	progeny	C	A	A	C	C/T
524A1	progeny	C	A/T	A/G	C/G	C/T
521B1	progeny	C/T	A	A	C	C/T
524C1	progeny	C/T	A/T	A/G	C/G	C
521D1	progeny	C	A	A	C	C
524B1	progeny	C/T	A	A	C	C/T
521F1	progeny	C/T	A	A	C	C
526B1	progeny	C	A	A	C	C
520C1	progeny	C/T	A	A	C	C/T
524D1	progeny	C/T	A/T	A/G	C/G	C
520J1	progeny	C	A	A	C	C
520K1	progeny	C/T	A/T	A/G	C/G	C
520R1	progeny	C	A/T	A/G	C/G	C
520H1	progeny	C/T	A/T	A/G	C/G	C/T
520I1	progeny	C	A	A	C	C/T
520P1	progeny	C	A	A	C	C
520W1	progeny	C	A	A	C	C
520X1	progeny	C	A	A	C	C
521C1	progeny	C	A	A	C	C/T
521E1	progeny	C/T	A	A	C	C/T
520T1	progeny	C	A/T	A/G	C/G	C/T
520S1	progeny	C	A/T	A/G	C/G	C
521W1	progeny	C/T	A	A	C	.
520G1	progeny	C	A	A	C	C
521K1	progeny	C/T	A	A	C	C
521L1	progeny	C	A/T	A/G	C/G	C/T
521M1	progeny	C	A	A	C	C/T
521N1	progeny	C	A/T	A/G	C/G	C/T
520U1	progeny	C/T	A/T	A/G	C/G	C
521I1	progeny	C/T	A/T	A/G	C/G	C
521T1	progeny	C/T	A/T	A/G	C/G	C
521P1	progeny	C	A/T	A/G	C/G	C
521G1	progeny	C	A/T	A/G	C/G	C
521U1	progeny	C	A	A	C	C/T
521V1	progeny	C/T	A/T	A/G	C/G	C/T
521Q1	progeny	C	A/T	A/G	C/G	C
521R1	progeny	C/T	A	A	C	C/T
521H1	progeny	C	A/T	A/G	C/G	C
520V1	progeny	C	A/T	A/G	C/G	C/T
526D1	progeny	C	A	A	C	C
520L1	progeny	C	A	A	C	C
520M1	progeny	C/T	A/T	A/G	C/G	C
526A1	progeny	C/T	A/T	A/G	C/G	C
521J1	progeny	C/T	A/T	A/G	C/G	C
520N1	progeny	C/T	A	A	C	C
521S1	progeny	C	A	A	C	C
520A1	progeny	C/T	A	A	C	C/T

Table 4.6 Typing scores of candidate markers associated with antibody titers to sheep red blood cells using radiation hybrid panel

Hybrid ID[a]		11	16	18	20	23	26	27	33	34	45	50	53	67	75	76	78	81	82	89	92	95	104	110	112	123	139	140	141	161	162
Marker ID	VTC01	0[b]	0	0	0	0	2[d]	0	0	1[c]	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	VTC02	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
	VTC03	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	VTC04	1	0	0	1	0	0	1	0	0	0	0	1	1	1	1	0	0	0	0	1	0	0	1	1	0	0	0	1	0	0
	VTC05	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	VTC06	0	0	1	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
	VTC07	0	0	0	0	0	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

Hybrid ID		166	173	175	177	178	179	182	190	197	207	237	243	253	274	285	286	305	307	310	318	327	331	333	337	350	364	365	366	373	375
Marker ID	VTC01	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
	VTC02	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	0	0	0	0
	VTC03	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	2	0
	VTC04	1	0	2	1	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	1	1	0	0	1	1	0	1	1	0	0
	VTC05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	VTC06	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	1
	VTC07	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0

Hybrid ID		377	386	397	476	482	483	511	523	524	531	536	541	546	557	566	567	575	576	587	588	593	594	600	608	611	613	618	628	635	639
Marker ID	VTC01	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	0	1	0	0	0
	VTC02	0	0	0	0	0	0	0	0	2	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
	VTC03	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
	VTC04	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	1	0	1	1	2	0	1	1	1	0	0	0	0	0	0
	VTC05	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	1	0	0	1	0
	VTC06	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	1	1
	VTC07	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Hybrid DNA used in the radiation hybrid panel.

[b, c,d] Scores 0, 1, and 2 indicate that the two PCR products for the same sample are absent, present, and ambiguous when positive in only one of the PCR reactions.

Table 4.7 Pairwise linkage disequilibrium (LD) analysis in backcross population

Locus1	Locus2	D'	LOD	Distance (bp)[a]
VTC01u10S2	*VTC01u10S1*	1.0	0.93	190
VTC01u10S2	*VTC01S2*	1.0	0.86	11885
VTC01u10S2	*VTC01S1*	1.0	12.05	11979
VTC01u10S2	*VTC01d5S1*	1.0	14.56	16711
VTC01u10S2	*VTC01d5S2*	1.0	4.63	16735
VTC01u10S1	*VTC01S2*	0.938	12.62	11695
VTC01u10S1	*VTC01S1*	1.0	0.82	11789
VTC01u10S1	*VTC01d5S1*	1.0	0.89	16521
VTC01u10S1	*VTC01d5S2*	1.0	7.98	16545
VTC01S2	*VTC01S1*	1.0	0.74	94
VTC01S2	*VTC01d5S1*	1.0	0.82	4826
VTC01S2	*VTC01d5S2*	0.908	4.85	4850
VTC01S1	*VTC01d5S1*	1.0	12.96	4732
VTC01S1	*VTC01d5S2*	1.0	5.04	4756
VTC01d5S1	*VTC01d5S2*	1.0	4.63	24
VTC02u10S1	*VTC02u10S3*	1.0	2.81	244
VTC02u10S1	*VTC02S5*	0.885	9.28	11208
VTC02u10S1	*VTC02S8*	0.902	12.91	11653
VTC02u10S1	*VTC02d10S1*	0.911	15.37	22469
VTC02u10S3	*VTC02S5*	1.0	2.15	10964
VTC02u10S3	*VTC02S8*	1.0	3.14	11409
VTC02u10S3	*VTC02d10S1*	1.0	2.9	22225
VTC02S5	*VTC02S8*	1.0	18.65	445
VTC02S5	*VTC02d10S1*	1.0	17.19	11261
VTC02S8	*VTC02d10S1*	1.0	22.36	10816
VTC03u10S1	*VTC03S1*	1.0	27.03	10901
VTC03u10S1	*VTC03d5S1*	1.0	24.78	17082
VTC03S1	*VTC03d5S1*	1.0	24.78	6181
VTC04u10S5	*VTC04u10S4*	1.0	0.88	37
VTC04u10S5	*VTC04u10S3*	1.0	0.88	175
VTC04u10S5	*VTC04u10S2*	1.0	2.41	207
VTC04u10S5	*VTC04u10S1*	1.0	3.99	264
VTC04u10S5	*VTC04S2*	1.0	3.8	10995
VTC04u10S5	*VTC04S1*	1.0	4.01	11114
VTC04u10S4	*VTC04u10S3*	1.0	13.42	138
VTC04u10S4	*VTC04u10S2*	1.0	1.51	170
VTC04u10S4	*VTC04u10S1*	1.0	1.68	227
VTC04u10S4	*VTC04S2*	1.0	1.79	10958
VTC04u10S4	*VTC04S1*	1.0	1.88	11077
VTC04u10S3	*VTC04u10S2*	1.0	1.51	32
VTC04u10S3	*VTC04u10S1*	1.0	1.68	89
VTC04u10S3	*VTC04S2*	1.0	1.79	10820
VTC04u10S3	*VTC04S1*	1.0	1.88	10939
VTC04u10S2	*VTC04u10S1*	1.0	17.26	57
VTC04u10S2	*VTC04S2*	0.911	13.4	10788
VTC04u10S2	*VTC04S1*	0.872	12.62	10907

Continued

VTC04u10S1	*VTC04S2*	0.966	23.44	10731
VTC04u10S1	*VTC04S1*	0.965	22.62	10850
VTC04S2	*VTC04S1*	1.0	29.13	119
VTC05u5S1	*VTC05S1*	1.0	2.74	5406
VTC05u5S1	*VTC05d10S1*	0.955	15.79	15151
VTC05S1	*VTC05d10S1*	1.0	3.27	9745
VTC06u10S2	*VTC06u10S1*	1.0	19.96	60
VTC06u10S2	*VTC06S2*	0.877	3.06	11428
VTC06u10S2	*VTC06d5S1*	0.861	2.61	16808
VTC06u10S2	*VTC06d5S2*	0.853	2.42	17000
VTC06u10S1	*VTC06S2*	1.0	4.22	11368
VTC06u10S1	*VTC06d5S1*	1.0	3.75	16748
VTC06u10S1	*VTC06d5S2*	1.0	3.56	16940
VTC06S2	*VTC06d5S1*	1.0	2.43	5380
VTC06S2	*VTC06d5S2*	1.0	2.08	5572
VTC06d5S1	*VTC06d5S2*	1.0	20.66	192

[a] the distance in base pair is between two SNPs.

Table 4.8 Case-control-based association analyses of each SNP and haplotype with anti-SRBC titers in the backcross population.

		Association tests[a]			permutation tests[b]	
Name	Alelle[c]/Hapl.(Freq.)[d]	Case, Control Ratios	Chi square	*p*-value	Chi Square	*p*-value
(BC2)						
VTC01u10S2	A	26:0, 28:6	5.098	0.024	5.098	0.0760
VTC01S1	T	26:0, 27:7	6.06	0.0138	6.06	0.0220
VTC01d5S1	A	26:0, 26:6	5.438	0.0197	5.438	0.0330
(BC2)						
VTC02d10S1	T	26:0, 31:5	3.928	0.0475	3.928	0.0730
(BC2)						
VTC03u10S1	A	21:5, 20:14	3.279	0.0702	3.279	0.0520
VTC03S1	G	21:5, 20:14	3.279	0.0702	3.279	0.0520
VTC03d5S1	G	21:5, 19:15	4.106	0.0427	4.106	0.0310
*VTC03*Block 1	AGG(0.667)	21.0 : 5.0, 19.0 : 15.0	4.106	0.0427	4.106	0.0310
*VTC03*Block 1	GAC(0.317)	5.0 : 21.0, 14.0 : 20.0	3.279	0.0702	3.279	0.0520
(BC1)						
VTC04u10S4	A	43:1, 14:4	6.857	0.0088	6.857	0.0300
VTC04u10S3	T	43:1, 14:4	6.857	0.0088	6.857	0.0300
VTC04u10S2	G	34:10, 7:11	8.403	0.0037	8.403	0.0070
*VTC04*Block1	GTG(0.661)	34.0 : 10.0, 7.0 : 11.0	8.403	0.0037	8.403	0.0070
*VTC04*Block1	ATG(0.081)	1.0 : 43.0, 4.0 : 14.0	6.857	0.0088	6.857	0.0300
(BC1)						
VTC06d5S1	A	27:17, 5:13	5.77	0.0163	5.77	0.0150
VTC06d5S2	T	26:16, 5:13	5.876	0.0153	5.876	0.0150
*VTC06*Block1	AT(0.516)	27.0 : 17.0, 5.0 : 13.0	5.77	0.0163	5.77	0.0150
*VTC06*Block1	TG(0.484)	17.0 : 27.0, 13.0 : 5.0	5.77	0.0163	5.77	0.0150

[a] The case-control association analyses of each SNP and haplotype with anti-SRBC titers were done in two types of backcross populations, BC1 and BC2, which were generated by backcrossing F_1 to the high and low anti-SRBC lines, respectively. Anti-SRBC titers were dummied into two values, '1' for 'Control' and '2' for 'Case'. Individuals with anti-SRBC titers >9 in BC_1 or <6 in BC_2

were labeled as ‘Case’, while the rest were labeled as ‘Control’. A total of 64 individuals from 26 families in the backcross were used. Significance of association was estimated by Chi-square test.

[b] Permutation test also was performed in order to obtain a measure of significance corrected for multiple testing bias. In the present experiment, the number of permutation was 1000.

[c] The allele for each SNP used for association study.

[d] Haplotype (Hapl.) was constructed using Haplotype3.32 (http://www.broad.mit.edu/mpg/haploview/). The haplotypes for each marker were visualized in Figure 4.7. The frequency (freq.) of each haplotype also was calculated and indicated.

Table 4.9 Candidate genes located near each candidate DNA marker

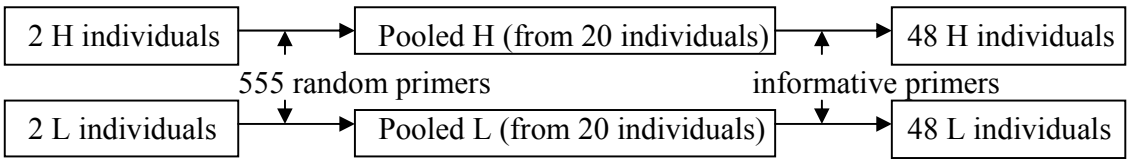
Marker	Candidate gene[a]	Annotation[b]	Distance (kbp)[c]
VTC01	*TCF7*	transcription factor 7, T-cell specific	1,145
	LTC4S	leukotriene C4 synthase	1,242
	CD74	class II invariant chain	1,644
VTC02	*C1QTNF7*	C1q and tumor necrosis factor related protein 7	111
	LOC422846	similar to Ly1 antibody reactive clone	1,902
	CYTL1	cytokine-like 1	2,250
VTC03	*LOC420912*	cadherin 12	129
	Cadherin	cadherin	549
	CDH10	cadherin 10	1,286
VTC04	*LOC428465*	similar to T-cell receptor gamma Vg2-Jg2	1,085
	LOC428464	similar to T-cell receptor gamma Vg2-Jg2	1,125
	RCJMB04_3g3	integrin, alpha 9	1,328
	LOC420756	similar to Integrin alpha9	1,386
	LOC420755	similar to Integrin alpha9	1,470
VTC05	*BTG1*	B-cell translocation gene 1, anti-proliferative	140
	SOCS2	suppressor of cytokine signaling 2	350
VTC06	*BCL10*	B-cell CLL/lymphoma 10	2,122
VTC07	*CD99*	similar to T-cell surface glycoprotein E2 precursor	1,635

[a] Genes with their official symbols near each SCAR marker identified in this study were assigned as candidate genes, because they are related to immune response based on previous literature and on annotations retrieved from GenBank.

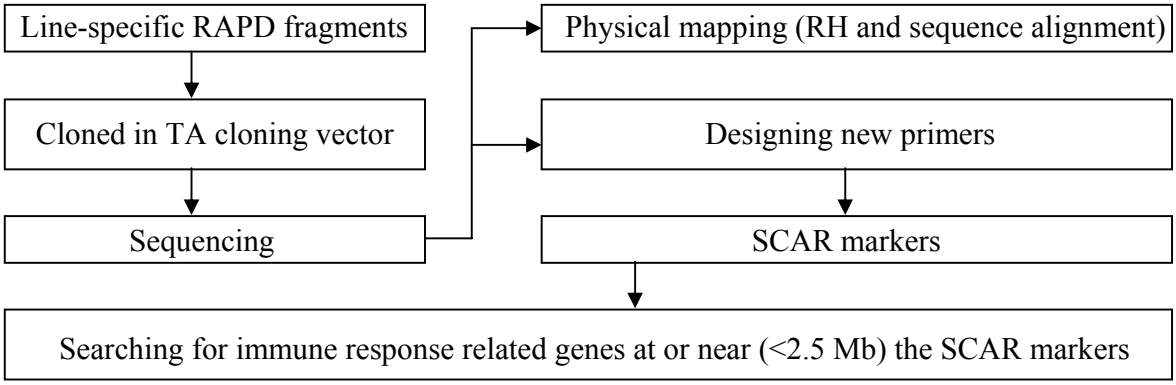
[b] The annotations were retrieved from *Gallus gallus* genome Build1.1 released in GenBank.

[c] The smallest distance in kilobase pair (kbp) between candidate marker and candidate gene.

A)



B)



C)

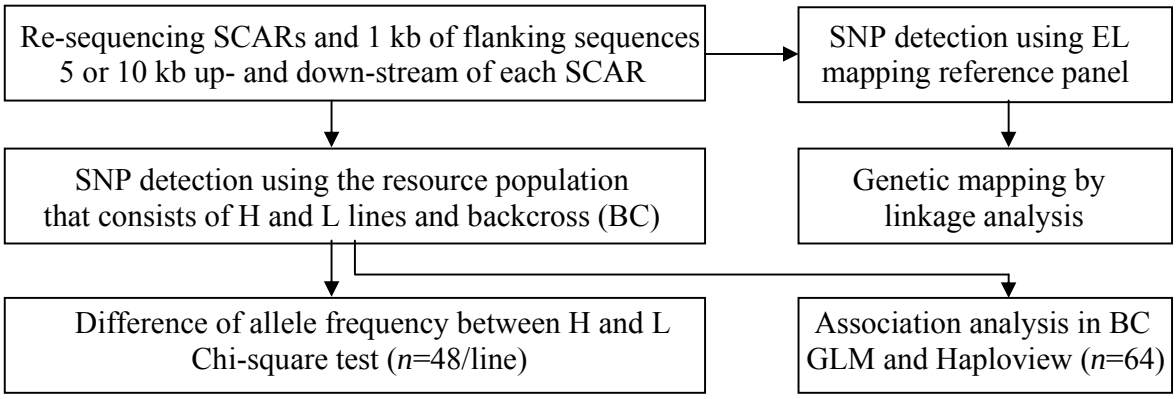


Figure 4.1 Summary of procedures in the study of RAPD- and SCAR-based QTL analysis for anti-SRBC response. A) Screening for random primers that amplify line-specific RAPD fragments. B) Converting RAPD fragments into sequence characterized amplified region (SCAR) markers. C) SNP analysis.

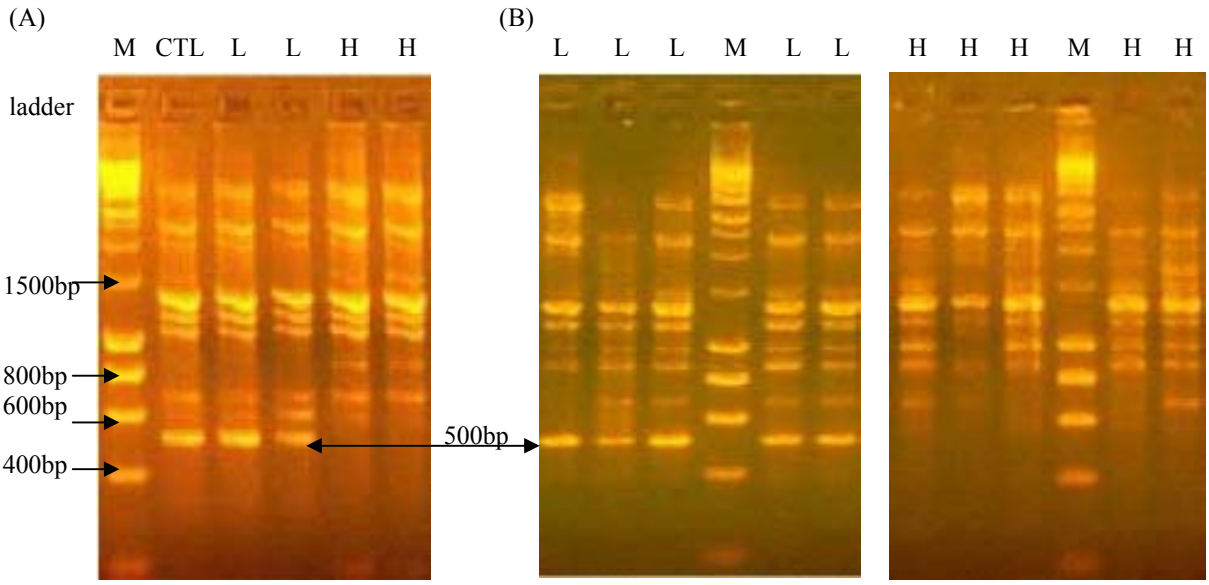
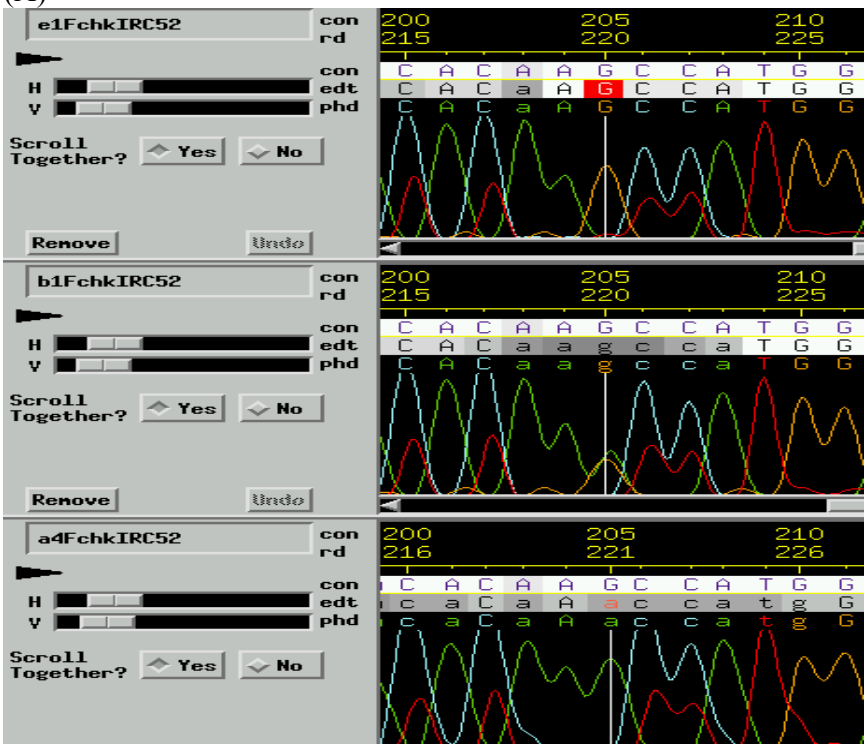


Figure 4.2 Line-specific RAPD fragment detected within (A) DNA pools and (B) individual samples from chickens with low (L) and high (H) antibody response using random primer Hokie3. The control (CTL) DNA sample was made by mixing equal amounts of DNA from the two DNA pools. M: DNA ladder.

(A)



(B)

Figure 4.3 A CONSED-generated trace (A) and alignment (B) showing a single nucleotide polymorphism (A/G) at position 205 (vertical white lines in trace A) detected in a candidate DNA marker, *VTC02*, in the parental lines. The sample IDs are shown on the right of panel B, with the first letter 'a' to 'd' for H line and 'e' to 'h' for L line.

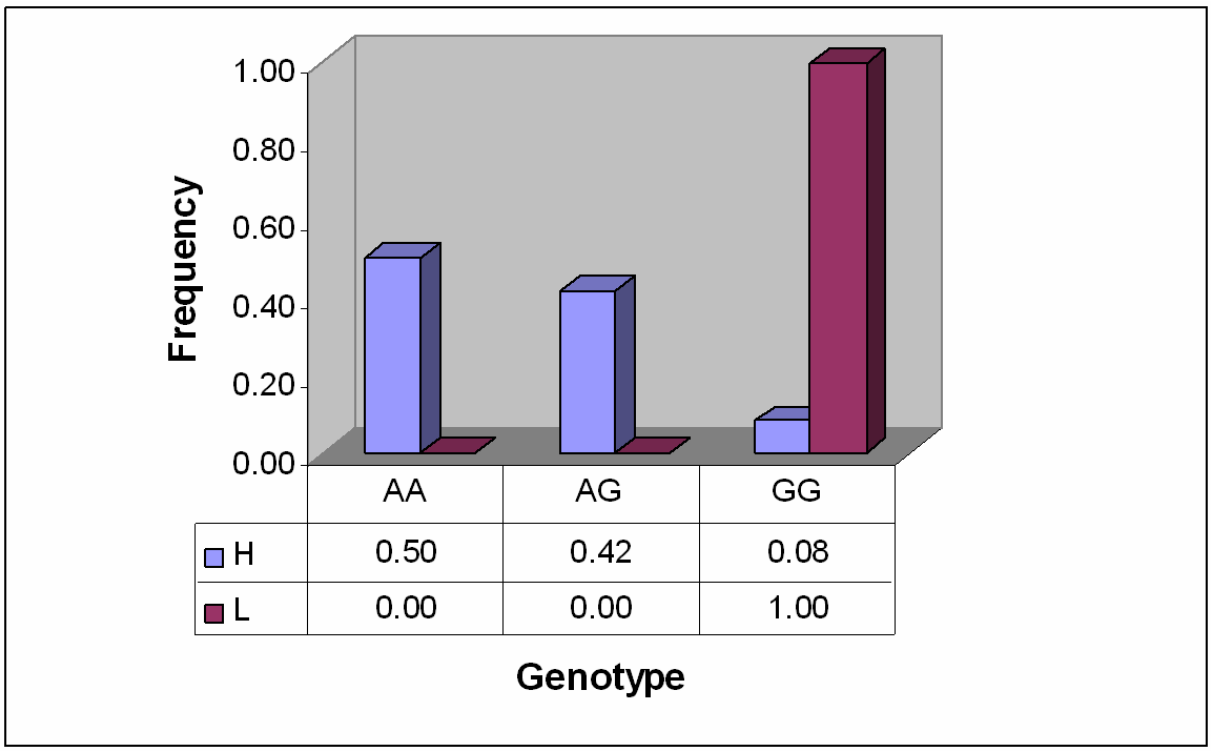


Figure 4.4 Genotype frequencies of the representative SNP at *VTC02* varying with different phenotypes - high (H) and low (L) antibody titers.

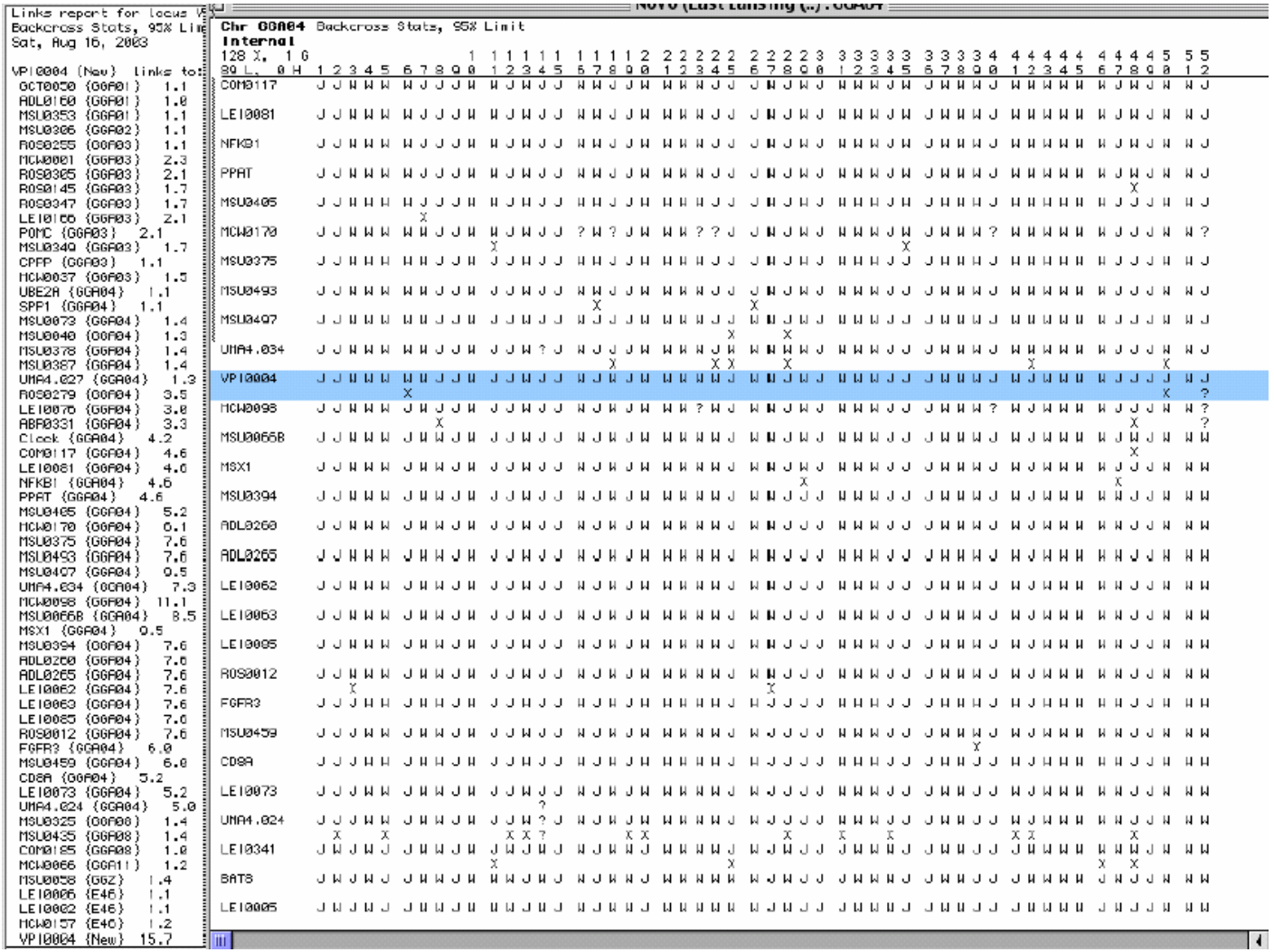


Figure 4.5 The chromosomal location of marker *VTC02* (or *VPI0004*). This marker was mapped between *MCW0098* and *UMA4.034*, with LOD scores of 11.1 and 7.3, respectively.

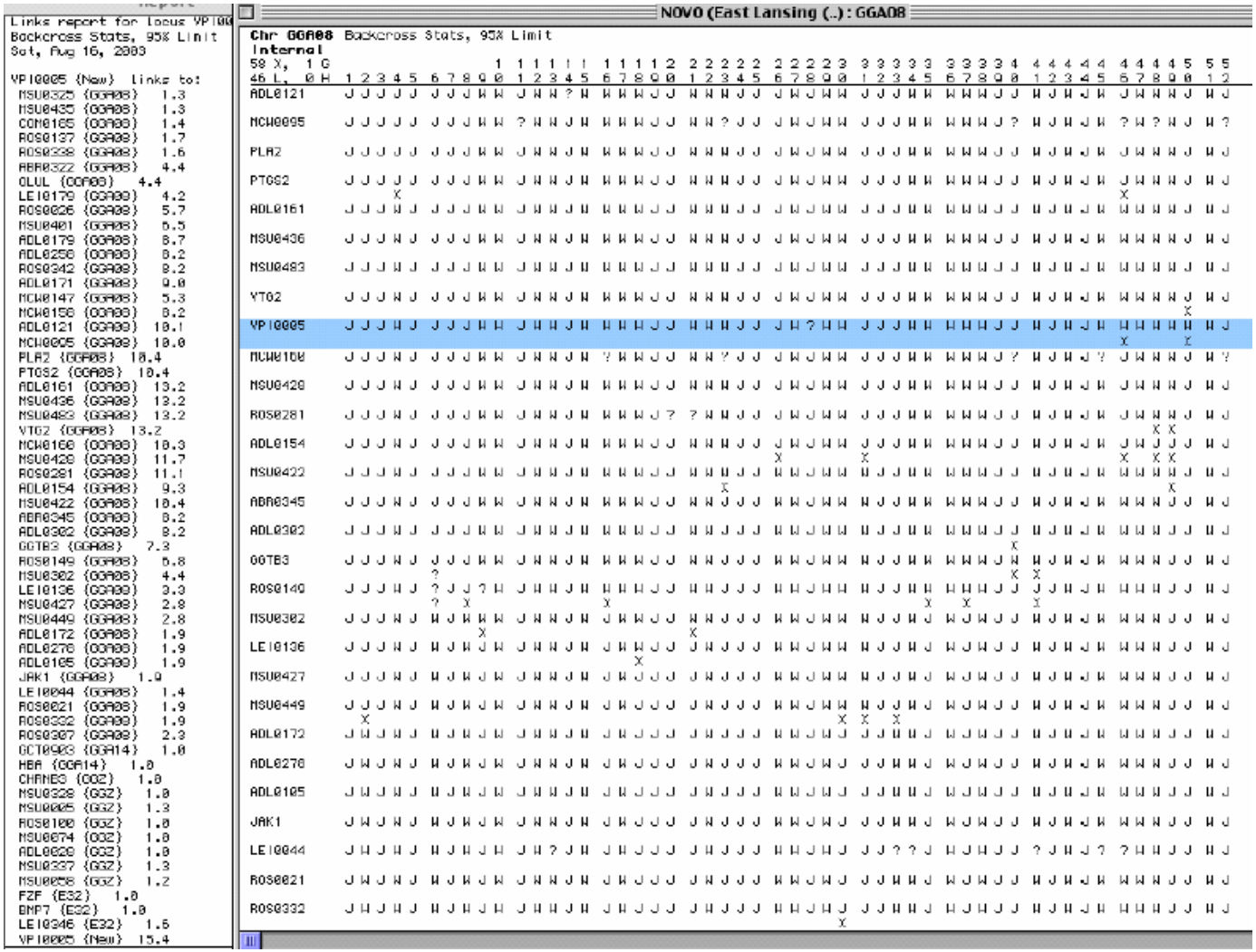


Figure 4.6 The chromosomal location of marker *VTC06* (or *VPI0005*). This marker was mapped between *VTG2* and *MCW0160*, with LOD scores of 13.2 and 10.3, respectively.

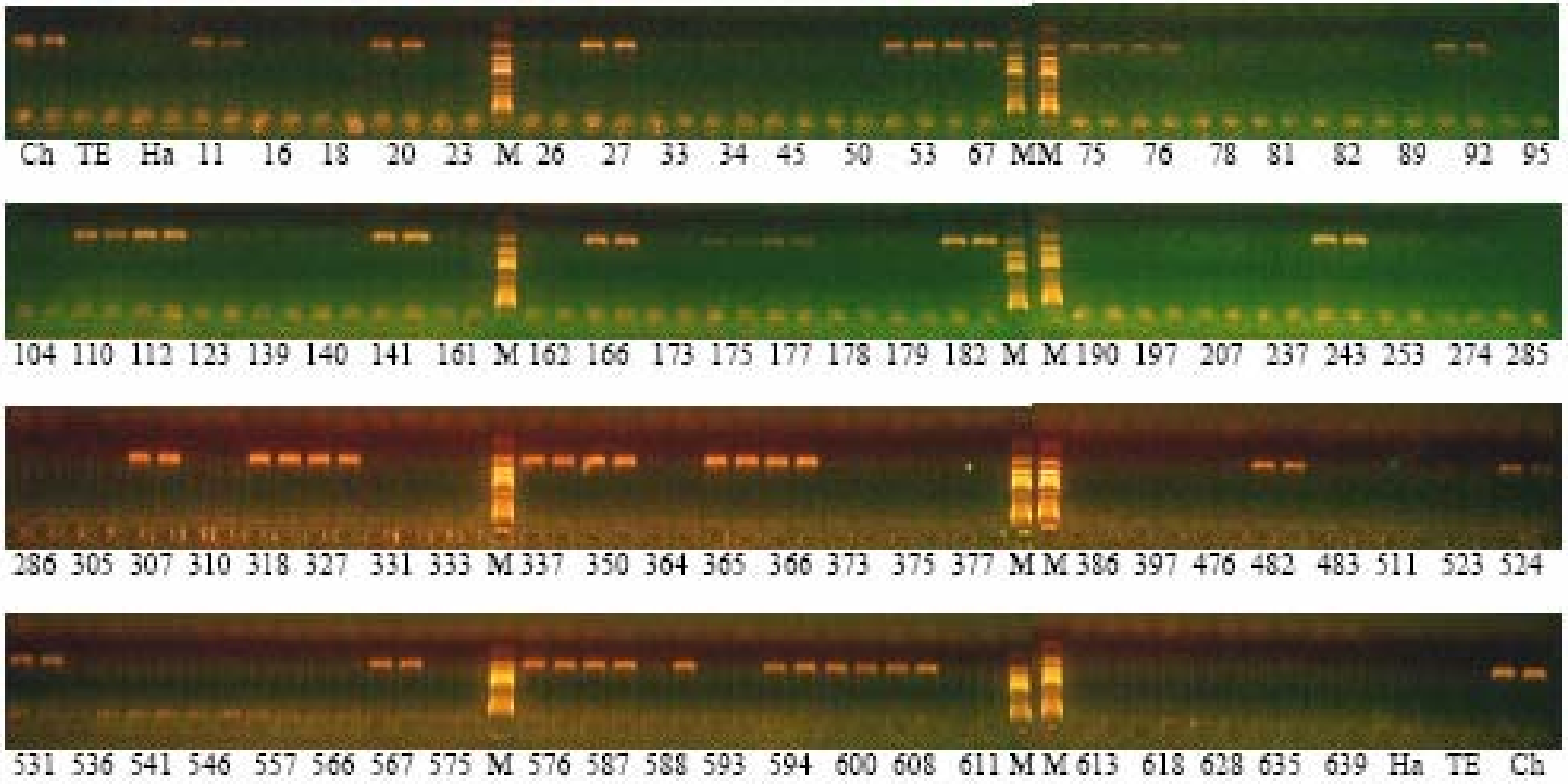


Figure 4.7 Sample of gel pattern for radiation hybridization (RH) mapping of one candidate marker, *VTC04*, using the RH panel developed by Morisson et al. (2002). The letters and numbers indicate the identities of hybrid DNA used in the RH panel. Ch, TE and Ha represent chicken DNA, TE buffer, and Hamster DNA, respectively. Two PCR reactions are performed separately for each hybrid DNA, and are loaded close to each other to make the analysis easier. Typing scores of each marker are recorded for mapping the location of the marker. The typing scores 0, 1, and 2 indicate that the two PCR products for the same sample are absent, present, and ambiguous when positive in only one of the PCR. M, the DNA ladder.

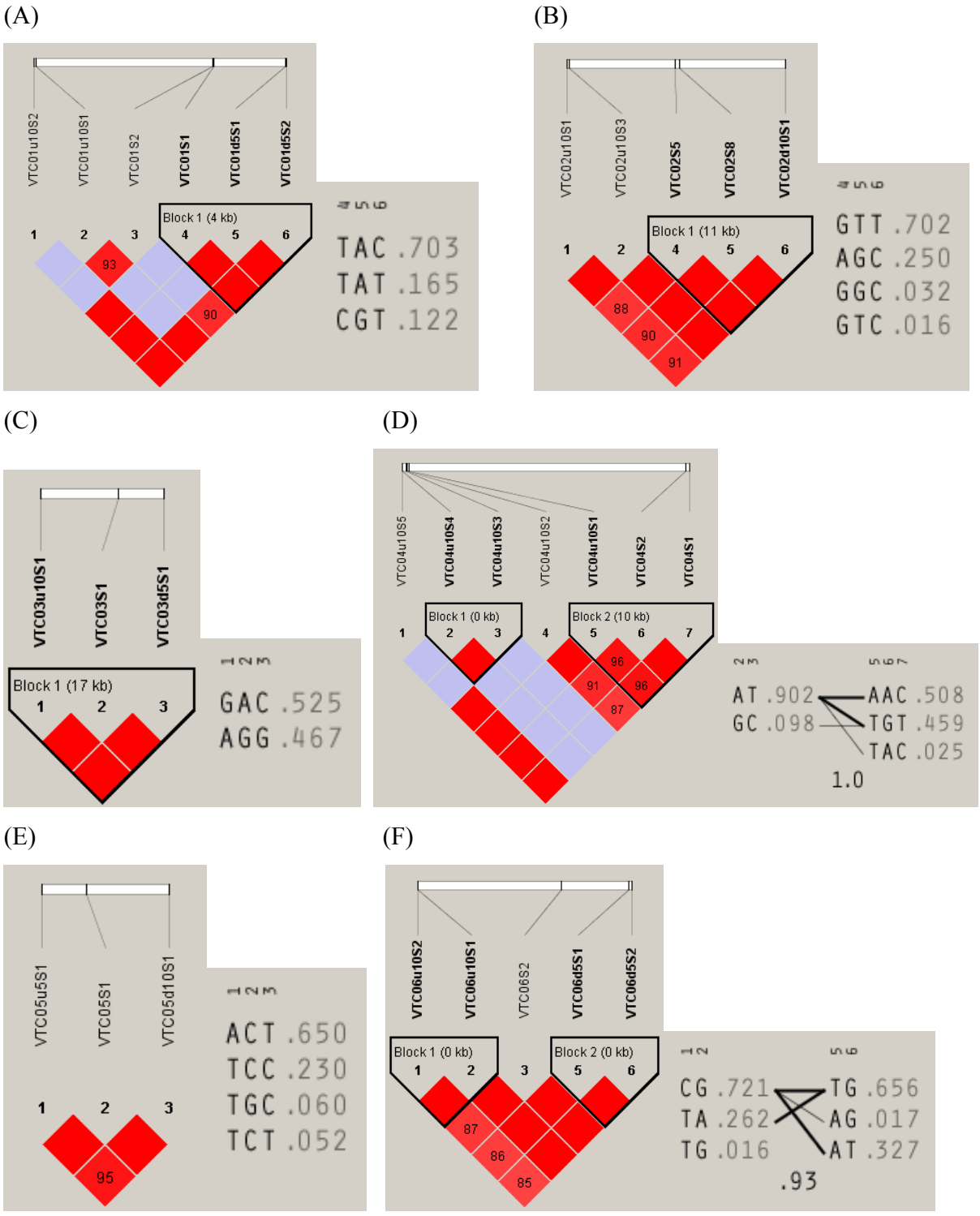


Figure 4.8 Pairwise linkage disequilibria (LDs) between SNPs within each candidate marker and flanking sequences are plotted by Haploview3.32 (http://www.broad.mit.edu/mpg/haploview/) and shown in panels A to F for *VTC01* to *VTC06*, respectively. The haplotype block(s) for each

marker constructed by Haploview3.32 using genotype and pedigree information from the backcross population are also shown beside the LD plots. In each LD plot, the white bar represents one of the chicken chromosomes, marked by the SNPs within the candidate marker and flanking sequences based on their locations on v2.1 draft assembly of chicken genome, which were determined by using genome browser tool-BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start). The color scheme for the LD plot is as follows: blue for D'=1 with LOD<2, bright red for D'=1 with LOD ≥2, and shades of red for D'<1 with LOD ≥2. The number on the shades of red indicates the D' value for the pairwise LD between the SNPs. Each haplotype is composed by one allele of the SNPs contained in the block, with its frequency beside.

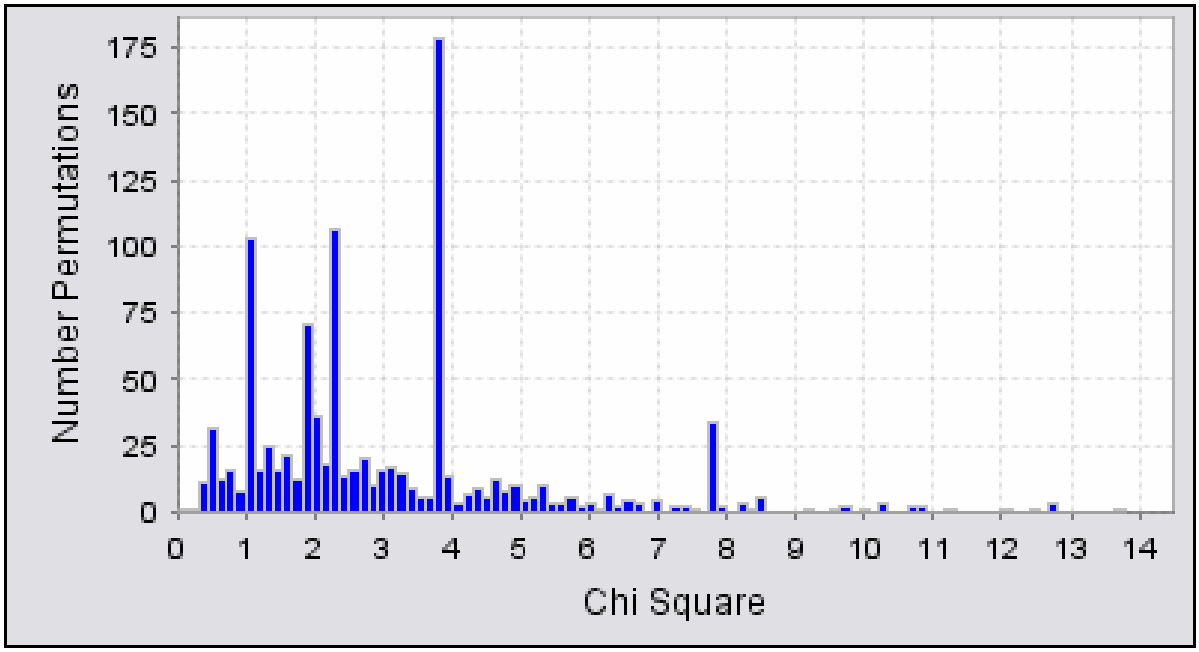


Figure 4.9 An example of a permutation test on the case-control association analysis of each marker and haplotype with anti-SRBC titers. In this example, a permutation Chi-square was estimated for *VTC03*, which is compared with the observed Chi-square to determine the *p*-values for the marker.

CHAPTER 5

RFLP-based analysis of candidate markers for antibody production in different populations

5.1 Abstract

The genetic improvement of livestock and poultry for natural disease resistance is an important goal in animal production. Obviously, at least two benefits can be gained from raising animals with strong natural disease resistance. The first is the direct improvement of animal health, while the second involves the indirect improvement of human health through minimizing the use of antibiotics in animal production. Often, however, disease resistance is a function of many genes that contribute to general or specific immune response to pathogens. Therefore, identification of quantitative trait loci associated with immune response can facilitate the accomplishment of this goal. Previously, I identified seven marker loci putatively associated with anti-SRBC response in chickens. Here, I describe a PCR-RFLP method for genotyping chickens for SNPs shown to segregate with anti-SRBC titers. Thirty birds from each of seven random-bred populations, including 3 non-commercial strains and 4 commercial lines of chickens, were genotyped for a selected SNP at 6 loci. The allele frequency distributions of the 6 SNPs in the the populations that were not directly selected for anti-SRBC provide additional support that the association of candidate SNPs with anti-SRBC shown in previous study (Chapter 4) may not be due to the effect of genetic drift. This study provides practical methods for future utilization of these loci to evaluate birds for immune response.

Key words: Antibody response, PCR-RFLP, SNPs, *Gallus gallus*

5.2 Introduction

Genetic markers have applications in evaluation of genetic diversity, QTL mapping, marker-assisted selection, phylogenetics and other marker-based genomic studies. One factor that affects their applications is the cost and time taken in genotyping genetic markers. A variety of methods have been developed for genotyping SNP markers, including sequencing, single-stranded conformational polymorphism, pyrosequencing, melting temperature statistics (denaturing-HPLC), *in silico* EST data mining, microarray-based genotyping, and PCR-RFLP. PCR-RFLP is one of the cost-effective and time-effective methods for SNP genotyping.

Another factor that affects the utility of a genetic marker is the universality of the genetic marker among different populations. Chicken breeds were developed for different purposes and were subject to both natural and artificial selection. They generally have large phenotypic and genetic differentiation among them. The universality of a genetic marker could be affected by the differential selection. The universality could be evaluated by determining the allele distributions of genetic markers in the populations.

I previously identified seven markers associated with anti-SRBC response in the White Leghorn chickens divergently selected for high or low anti-SRBC titers. These genetic markers may be used for improving natural disease resistance because evidence indicated that antibody production was associated with disease resistance of vertebrates (Gross et al., 1980, 2002). Here, a PCR-RFLP method for genotyping a selected SNP at each locus was developed. The universality of each candidate genetic marker was evaluated in 7 random-bred populations, including 4 commercial strains and 3 traditional breeds, using the PCR-RFLP method. The PCR-RFLP method developed here allows relatively rapid and less costly genotyping of chickens for the variant nucleotides.

5.3 Materials and Methods

5.3.1 Animals and genomic DNA

Four commercial lines and 3 traditional breeds of chicken were used in this experiment. The commercial strains are ISA-A, ISA-C, ISA-D and Cobb500, while the traditional breeds include Rhode Island Red (RIR), White Plymouth Rock (WPR) and Araucona. All populations except Cobb500 have previously been described (Smith et al., 2002). Cobb500 is a commercial broiler line from Tyson Foods Company. Genomic DNA from 30 individual birds of each population, isolated from red blood cells by the protocol previously described (Smith et al., 1996), was used in the PCR-RFLP analysis at each marker locus.

5.3.2 Candidate markers associated with anti-SRBC titers

The six candidate markers associated with anti-SRBC titers were previously identified in the lines divergently selected for high or low anti-SRBC titers (See Chapter 3 and Chapter 4). Each of them was derived from a unique RAPD fragment that was subsequently sequenced by a standard method. Each sequence was submitted to GenBank and has been assigned Accession Numbers (Table 5.1). The chromosomal location of each sequence was determined using BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat) to search the recently-released chicken DNA sequence (Table 5.1).

Primers were designed from each of the marker sequences using the Primer 3 software (Rozen and Skaletsky, 2000). The sequences of the primers for each locus, presented in Table 5.1, were used to produce an amplicon of 200 to 600 bp. PCR was performed in a total of 25 µl using 50 ng of genomic DNA, 1× standard PCR buffer containing 2 mM $MgCl_2$ (pH 8.7), 200 µM each dNTP, 50 ng of each primer and 2.5 U of *Taq* DNA polymerase (Fisher Scientific, Inc.

MO). Amplification was carried out in a Mastercycler (Brinkmann, Westbury, NY) under the following cycling conditions: 95°C for 5 min for initial denaturation; 38 cycles of 95°C for 45 sec, annealing temperature that ranged from 60°C~64°C for 45 sec, and 72°C for 45 sec; and a final extension at 72°C for 7 min.

5.3.3 Restriction fragment length polymorphism

Single nucleotide polymorphisms within each marker were previously identified in the resource population (See Chapter 4). Only those located within each amplicon mentioned above were used to scan for the presence of a naturally-occuring restriction site using the web-based program Cutter (http://www.justbio.com). For each locus, 10 μL of PCR product was digested in a final volume of 15 μL with 10 U of the restriction enzyme and incubated at 37ºC for 4 hours. The digested PCR product was separated by regular electrophoresis with a 2% agarose gel containing SYBR green dye (Molecular Probes, Inc.). Based on the digestion pattern (Figure 5.1), the genotype of individual birds were determined and recorded. Allele frequencies in each population of chicken then were calculated.

5.4 Results and Discussion

Presented in Figure 5.1 is an example of the restriction patterns observed from the PCR-RFLP analysis at each locus. Allele frequencies of each candidate marker are shown in Table 5.1. The estimates of variation in the 7 populations were similar to those previously reported (Smith et al., 2000) using re-sequencing data and SNP analysis of ESTs from different loci. On average, 2.7 of the 6 markers were not or were minimally polymorphic for each population and 2.3 of 7 populations were not or were minimally heterozygous for each marker. The degree of polymorphism of the markers in these populations ranked from low to high is as follows: *VTC06,*

VTC01, VTC04, VTC07, and (*VTC02, VTC03*). The degree of heterozygosity of the populations is arranged from low to high as follows: White Plymouth Rock, (ISA-A, ISA-D and Cobb500), Rhode Island Red (RIR), ISA-C, and Araucona (data not shown).

The loss of polymorphism or heterozygosity at the candidate marker loci in these populations might reflect a breeding history in which antibody production was selected upon as a correlated response to selection for other traits. This inference is supported by the observations that selection on anti-SRBC titers was correlated with selection on production traits and disease resistance in the chicken (Pinard-van der Laan et al., 1998; Lamont et al., 2003; Kean et al., 1994). Because the utility of a marker is dependent on its polymorphism in a population, the data for the candidate markers suggested that the markers could prove useful in marker-assisted selection for antibody response in only some populations provided associations of the markers with antibody response are confirmed. In other words, the evaluation on the universality of the candidate markers in this study provides information useful for possible future use. In addition, because PCR-RFLP represents a rapid and cost-effective tools for genotyping a population, the established PCR-RFLP methods for the candidate markers would increase the utility of the markers in a marker-assisted breeding program for immune response.

Table 5.1 Characteristics and population frequency distribution of variant nucleotides in six chicken DNA sequences.

Sequence ID	Accession Number/ size[1]	Chromosomal location [2]	Primers	Restriction Endonuclease[3]	SNP(RFL)[4]	Allele Frequency[5]						
						A	C	D	Cobb	RIR	WPR	Arau
VTC01	AY435424/ 207	Chromosome 13: 15192367-15193230	F:aaccccagcttgaaaacaga R:gccagctgtctgcttaacac	*HphI*	*A*,G (41,166)	0.02	0.32	0.05	0.68	0.00	0.02	0.70
VTC02	AY435425/ 576	Chromosome 4: 79574744-79575886	F:ttggaaagcttggcactttt R:ctgctcgtgcaatcagaaga	*EcoRI*	*A*,C (166,415)	0.27	0.25	0.65	0.23	0.35	0.00	0.27
VTC03	AY435426/ 258	Chromosome 2: 74967060-74967568	F:ctgcagtgcaaaaggttcac R:aattggagcgagacagatgg	*Hpy8I*	*A*,G (66,177)	0.37	1.00	0.57	0.38	0.55	0.46	0.70
VTC04	AY435427/ 265	Chromosome 2: 50346631-50347236	F:aaggctgtaaggctgtaacaca R:gaatcttgagaggttcacacagg	*Csp6I*	*C*,T (41,187)	0.77	0.32	0.00	0.02	0.10	0.00	0.30
VTC06	AY435429/ 358	Chromosome 8: 19189926-19190404	F:aggagacctgtgcagaatgg R:atgttctttcatgcatcccc	*BmfI*	*C*,T (82,276)	0.02	0.10	0.00	0.02	0.02	0.04	0.32
VTC07	AY435430/ 389	Chromosome 1: 131019193-131019716	F:ccaaactacccaaaagaggg R:agagagcaggtgtgagaagc	*Hpy188III*	*C*,T (30,124)	1.00	0.73	0.37	0.00	0.08	0.37	0.57

[1] Genbank accession number and size in base pairs of the DNA sequence.

[2] BLAT- based chromosomal assignment of the DNA sequence.

[3] Restriction endonuclease used for digestion of the DNA sequence. The recognition sequence of each of these enzymes was natural at the SNP location.

[4] The alleles of the SNP at the restriction enzyme site, RFL refers to the size, in base pairs, of the fragments produced from the restriction digestion.

[5] Frequency of the italicized SNP allele in the seven populations which include 30 individual birds for each. A, C, D, Cobb, RIR, WPR and Arau represent commercial lines (ISA A,C and D) from Merial Ltd. Company, commercial line Cobb500 from Tyson Foods company, Rhode Island Red chicken, White Plymouth Rock and Araucona, respectively.

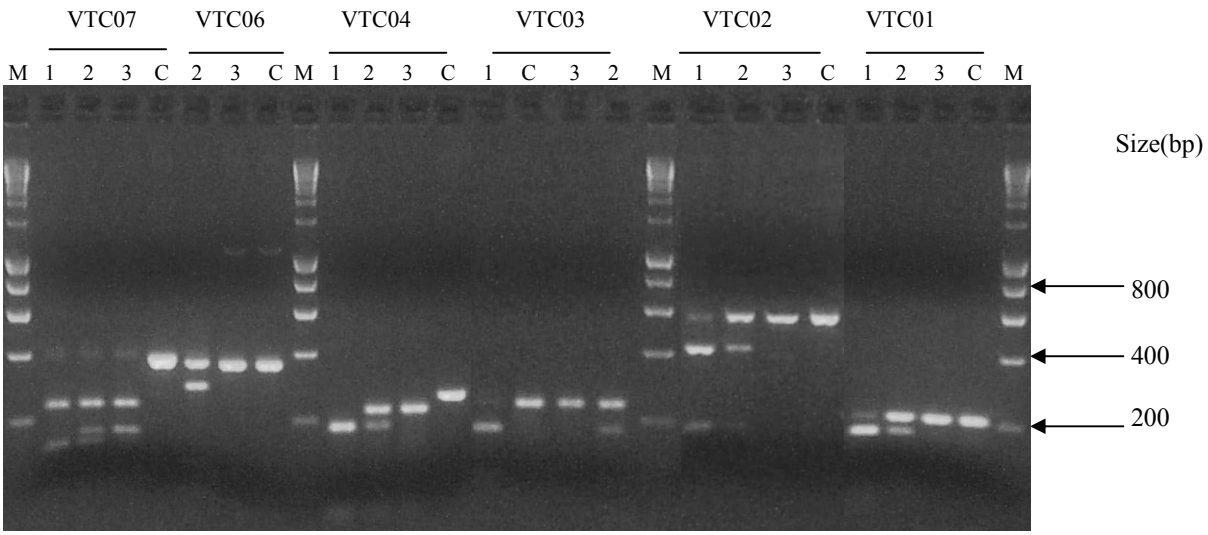


Figure 5.1 SYBR green-stained agarose gel patterns of PCR-RFLP genotypes showing the variant nucleotides at each of the 6 loci analyzed. The digested patterns labeled as *VTC01-07* were generated from digestions of PCR products using enzymes described in Table 1. Lanes identified as M and C represent 1 Kb DNA ladder and control (undigested PCR product from each locus) samples, respectively. For each locus, lanes labeled as 1, 2, and 3 represent a digested pattern of an amplicon of a homozygote (restriction site on both chromosomes), heterozygote (restriction site on only one chromosome) and homozygote (no restriction site) bird, respectively.

CHAPTER 6

Microarray-based SNP analysis of chickens identified as either high or low anti-SRBC response

6.1 Abstract

Microarrays provide a powerful tool to conduct a genome-wide scan for candidate DNA markers associated with a quantitative trait. Here, the Illumina SNP analysis array was used to search for SNPs associated with anti-SRBC response in the chicken. Because of cost limitation, only 9 birds, including 4 identified as low antibody producing and 5 as high, were used. A total of 2,733 SNPs were identified and validated. Fifty-seven of the informative SNPs appeared to be line-specific and were mostly located on different chromosomes including chromosome 1 (8 SNPs), 2 (9), 4 (6), 15 (4), 16 (4), and Z (11). Twenty of the line-specific SNPs were within or near (<2 Mb) the genes previously reported to have immune-related function. In addition to the novel candidate markers, the results appeared to validate 6 of 7 candidate markers previously identified by us through other genome analysis methods. These findings may provide a useful resource for identifying genes that control humoral immunity in chickens.

Key words: Single nucleotide polymorphism, humoral immune response, *Gallus gallus*

6.2 Introduction

Heritable variations analyzed through DNA analysis provide the genomics community with a resource that facilitates phenotype:genotype assignment. A major resource in this effort is expected to be single nucleotide polymorphisms (SNPs). SNPs are abundant in most genomes, including the chicken, where they occur at a frequency of 1 every 470 bp (Smith et al., 2000 and Wong et al., 2004). In humans, it has been estimated that the SNP frequency is 0.05% to 0.1% in genes, though higher in intergenic regions and introns. Several efforts for SNP discovery have been described, yielding 2.8 million SNPs in the chicken (Wong et al., 2004), in the human (Wang et al., 1998) and in other species. Besides their abundance, SNPs have become DNA markers of choice for the third generation of genome maps because of their compatibility with automation and high throughput analysis. This compatibility facilitates high-resolution genome-wide scans for genes underlying various biological traits, especially polygenic traits. In addition, because non-synonymous SNPs located in coding sequences in many genomes, including chicken, have been sequenced and annotated, investigations with these SNPs may have the advantage of supporting a candidate-gene study.

The interest in LD-based genome-wide association studies using highly dense arrays of SNPs is increasing. One reason is that association studies have greater power than linkage analysis in detecting genes or QTLs underlying polygenic traits, especially those with small effects on the traits (Risch and Merikangas, 1996). Use of divergently selected lines for a polygenic trait may facilitate QTL identification since selection would make QTLs associated with the trait more detectable by causing allele frequency differentiation among the lines. That is, QTLs could be identified through LD-based approaches based on frequency changes of marker alleles under selection, although some limits should be considered and carefully checked prior to

conclusions (Keightley and Bulfield, 1993). Experimental lines divergently selected for anti-SRBC have been established (Siegel and Gross, 1980; Van der Zijpp et al., 1983). These lines provide a unique resource for identification of QTLs influencing anti-SRBC titers.

Antibody response is one of the important mechanisms by which vertebrates fight pathogens. Evidence has shown that the capacity of animals for anti-SRBC response is associated with natural disease resistance (Gross et al., 1980, 2002). Identification of QTL associated with anti-SRBC response may have implications for genetic improvement of disease resistance. Genetic control of anti-SRBC is polygenic (Feingold et al., 1976; Biozzi et al., 1979; Gross and Siegel, 1980). Several QTLs responsible for anti-SRBC titers were identified using lines of mice divergently selected for anti-SRBC response through linkage analysis (Puel et al., 1995, 1996). These QTLs not only included immunomodulatory genes at MHC and *Igh* loci, but also included some unknown immunomodulatory genes located on several distinct chromosomal segments. Similarly, Siwek et al. (2003) identified several markers associated with anti-SRBC response on chromosomes 3, 5, 6, 9, 10, 15, 16, 23 and 27 by linkage-based QTL analysis using F_2 birds produced by egg-laying chickens divergently selected for anti-SRBC response. However, the knowledge about QTLs influencing anti-SRBC response remains limited. In this paper, I study the distribution of QTLs associated with the trait on a genome-wide scale through LD-based analysis.

6.3 Materials and Methods

6.3.1 Animals and DNA samples

The establishment of genetic lines divergently selected for anti-SRBC titers was described in the Chapter 3. The 28th generation used in this experiment had mean anti-SRBC

titers of 11.5 and 2.6 for the H and L lines, respectively. Five unrelated individuals from the H line and four individuals from the L line were used. Genomic DNA was extracted from a whole blood samples collected from each individual using a protocol previously described (Smith et al., 1996). Each DNA sample was quantified and diluted to a concentration of 100 ng/ul. These samples were subsequently used for microarray-based SNP typing.

6.3.2 Microarray-based SNP analysis

A GoldenGate assay (Illumina, http://www.illumina.com/pages.ilmn?ID=11) developed by Illumina Corp. (San Diego, CA) was used. The microarrays used in this study were fabricated by Illumina Corp. Each microarray has a capacity for typing 3,072 SNPs. The probes on these microarrays were designed with input sequences downloaded from chicken SNP database. The input sequences are genomic DNA containing the SNPs, which were selected to be equally distributed throughout the chicken genome. The probes laid down on the microarray are oligos. Illumina Corp. performed the microarray-based SNP typing assays and reported the data to me. The procedures used for performing microarray experiment are described in GoldenGate Assay Workflow (www.snp-facility.med.uu.nl/pdf/goldengate_assay_final.pdf). The quality of these typing assays was evaluated by a number of procedures including sample success rate, locus success rate, call rate for genotypes, and reproducibility.

6.3.3 Bioinformatics analysis

After the typing assay, SNPs with only one allele per line were considered as candiate markers associated with anti-SRBC response. The immune response-related genes at or near (<2 Mb) each candidate marker was searched for using the locus name of the SNP to query against chicken SNP database in GenBank.

6.4 Results and Discussion

6.4.1 SNP typing assays

The sample success rate was 100%. The genotype call rate was 99.60%. The reproducibility was 100%. The locus success rate was 88.96%, meaning that 2733 of 3072 loci were successfully genotyped. These parameters collectively indicated high quality of microarray-based SNP typing assays.

6.4.2 SNPs associated with anti-SRBC titers

A total of 191 informative SNPs were identified as having at least one allele that was line-specific. Their distributions are shown in Table 6.1 and summarized in Table 6.3. They could be classified into three types of SNPs, including those located within or close to QTLs, not close to QTLs but being in LD with QTLs, and false positive. Among the informative SNPs, 57 appeared to be line-specific for both alleles (Table 6.2, Table 6.3). These SNPs might be strongly associated with anti-SRBC response. Bioinformatics analysis indicated that most of them were on chromosomes 1(8 SNPs), 2(9), 4(6), 15(4), 16(4), and Z(11). This distribution appeared to agree with my previous study (Chapter 4) in which 7 line-specific markers identified through RAPD-based approach were on the chromosomes 1(2 independent markers), 2(2), 4(1), 8(1), and 13(1). In fact, 6 of the 7 candidate markers were validated by this study (Table 6.2). This distribution also was supported by the study in which 2 independent QTLs influencing anti-SRBC titers were on chromosome 2 (Yonash et al., 2001). However, QTLs identified by Siwek et al. (2003) were on the chromosomes 3, 5, 6, 9, 10, 15, 16, 23 and 27, with 1 QTL per chromosome. Zhou et al. (2003) reported 4 QTLs associated with anti-SRBC response on chromosomes 3, 5, 6 and Z, separately. Results seem not to agree among studies about the distributions of most QTLs. This discrepancy is probably caused by use of different resource

population (e.g., White Leghorn vs. ISA, meat-type vs. egg-type) and approaches (e.g., linkage-based analysis vs. LD-based analysis) for QTL identification. Such a phenomenon was also observed in other animal species (Rupp & Boichard, 2003; Schulman *et al.,* 2004). In addition, since MHC and *Igh* genes reside on chicken chromosomes 1, 13, 15, 16, 17 and 18, as indicated by searching the chicken genome with Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/), the results of this study was also partially supported.

6.4.3 Candidate genes for anti-SRBC titers

The line-specific SNPs subsequently were used to search for immune response-related genes at or near them based on current annotation of chicken genome. This bioinformatics analysis indicated that 37 line-specific SNPs reside in known or hypothetical genes (Table 6.2), 20 of which were within or near (<2 Mb) immune response-related genes. In particular, some of these genes, such as MHC, toll-like receptors, complement component, and immunoglobulin, are known to involve antibody response (Malnati et al., 1992; Puel et al., 1996; Pieters, 2000; Mukhopadhyay et al., 2004; Reid and Porter, 1981; Fearon and Carroll, 2000; Butler, 1998; Rees and Nordskog, 1981). For example, snp-368-18-5079-S-3 was within a gene similar to class II histocompatibility antigen M-beta chain 1. Snp-29-306-157307-S-2 was near toll-like receptor 7. Snp-202-52-51925-S-1 resides in a gene similar to complement component 6.

In summary, the present study outlines the distributions of putative QTLs associated with anti-SRBC titers in chicken through LD-based analysis using divergently selected lines. It also provides a resource for future identification of causative genes. It is now clear that LD-based high-resolution whole-genome QTL analysis can prove successful (Ozaki et al., 2002; Klein et al., 2005; Maraganore et al., 2005), although the major locus sometimes could be missed (Conley et al., 2005; Rivera et al., 2005). Therefore, this preliminary study may be considered useful for

identification of QTLs associated with anti-SRBC response using a microarray-based genotyping approach. However, it is obvious that this study is very limited. First, only a small number of individuals from each line were examined. Second, the SNPs on the microarray were not haplotype-tagged SNPs. The SNP density in the Illumina chicken microarray was limited for determining precise locations of candidate genes. Last, the results were only based on frequency changes of SNPs and did not utilize any family-based information, which may lead to some false positive detections.

Table 6.1 Informative SNPs that had at least one allele that was line-specific.

Chr	Locus name	Allele		Genotype								
		A	B	H[b] (M[c])	H (M)	H (F[c])	H (F)	H (M)	L[b] (F)	L (F)	L (F)	L (M)
1	*snp-120-149-1449-S-2*	C	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
1	*snp-104-32-4713-S-3*	A	G	AB	BB	BB	AB	BB	AA	AA	AA	AA
1	*snp-169-85-6362-S-3*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
1	*snp-167-56-17685-S-3*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
1	*snp-6-599-22146-S-2*	A	C	BB	BB	BB	BB	BB	AA	AA	AA	AA
1	*snp-6-490-4449-S-1*	A	G	AB	BB	AB	AB	AB	AA	AA	AA	AA
1	*snp-6-336-15036-S-1*	A	G	BB	AB	AB	BB	AB	AA	AA	AA	AA
1	*snp-6-308-57894-S-3*	A	G	BB	BB	BB	BB	BB	AB	AA	AB	AA
1	*snp-6-244-21245-S-3*	A	G	BB	BB	BB	BB	BB	AB	AA	AA	
1	*snp-36-75-2425-S-2*	A	G	BB	AB	BB	AB	BB	AA	AA	AA	AA
1	*snp-36-82-43632-S-2*	A	C	AA	AB	AA	AB	AA	BB	BB	BB	BB
1	*snp-7-58-355-S-1*	A	G	BB	BB	BB	BB	BB	AA	AB	AB	AA
1	*snp-7-85-3009-S-1*	A	G	BB	BB	BB	BB	BB	AA	AB	AB	AA
1	*snp-7-171-6691-S-2*	C	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
1	*snp-7-194-27006-S-1*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
1	*snp-7-208-26246-S-2*	A	G	AA	AA	AA	AB	AB	BB	BB	BB	BB
1	*snp-7-278-21366-S-2*	A	G	BB	AB	BB	BB	BB	AA	AA	AA	AA
1	*snp-7-701-17073-S-3*	A	G	BB	BB	BB	BB	AB	AB	AA	AB	AB
1	*snp-56-112-23112-S-1*	A	G	AB	BB	BB	BB	BB	AA	AA	AA	AA
1	*snp-29-214-17765-S-3*	A	C	AA	AB	AA	AA	AA	BB	BB	BB	BB
1	*snp-29-292-10583-S-2*	A	G	AA	AB	AA	AA	AA	BB	BB	BB	BB
1	*snp-29-306-157307-S-2*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
1	*snp-287-27-28163-S-2*	A	G	BB	BB	BB	BB	BB	AB	AB	AA	AA
1	*snp-14-113-36609-S-1*	A	G	AA	AA	AA	AA	AA	AB	BB	BB	BB
1	*snp-14-60-77306-S-2*	A	C	AA	AA	AA	AA	AB	BB	BB	BB	BB
1	*snp-14-51-64946-S-1*	A	G	BB	BB	BB	BB	AB	AA	AA	AA	AA
1	*snp-23-159-68423-S-1*	C	G	AA	AA	AA	AA	AB	BB	BB	BB	BB
1	*snp-23-208-36692-S-1*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
1	*snp-23-272-12919-S-3*	A	G	AA	AA	AA	AA	AB	BB	BB	BB	BB
1	*snp-23-325-119425-S-2*	A	G	BB	BB	AB	BB	BB	AA	AA	AA	AA
1	*snp-23-342-18608-S-2*	A	G	BB	BB	AB	BB	BB	AA	AA	AA	AA
1	*snp-143-37-75034-S-2*	A	G	BB	BB	BB	BB	BB	AB	AB	AB	AA
2	*snp-2-682-159806-S-2*	A	G	AA	AA	AA	AA	AA	AB	AB	BB	AB
2	*snp-2-655-11730-S-3*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AB
2	*snp-2-637-25815-S-3*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	AB
2	*snp-2-569-7651-S-2*	A	C	BB	BB	BB	BB	BB	AA	AA	AB	AB
2	*snp-2-517-8510-S-3*	A	G	BB	BB	BB	BB	BB	AA	AB	AB	AB
2	*snp-2-31-74623-S-1*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	AB
2	*snp-5-177-14435-S-2*	A	C	BB	BB	BB	BB	BB	AA	AA	AA	AA
2	*snp-5-213-47708-S-3*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
2	*snp-5-236-875-S-1*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
2	*snp-5-296-38423-S-2*	A	G	AB	AA	AA	AA	AA	BB	BB	BB	BB
2	*snp-5-352-47450-S-2*	A	G	AB	AA	AA	AA	AA	BB	BB	BB	
2	*snp-5-355-27176-S-1*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
2	*snp-5-512-42242-S-3*	C	G	AB	AA	AA	AA	AA	BB	BB	BB	BB
2	*snp-5-524-143622-S-1*	A	G	AB	BB	BB	BB	BB	AA	AA	AA	AA
2	*snp-5-614-24216-S-3*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
2	*snp-40-25-19645-S-2*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	
2	*snp-40-38-1472-S-1*	A	G	BB	BB	AB	BB	BB	AA	AA	AA	
2	*snp-40-83-7191-S-2*	A	C	BB	BB	BB	BB	BB	AB	AB	AA	AA

2	*snp-40-160-66815-S-3*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
2	*snp-74-198-33913-S-1*	A	G	AA	AA	AA	AA	AA	BB	AB	BB	BB
2	*snp-210-32-2556-S-1*	A	C	AA	AA	AA	AA	AA	BB	AB	AB	BB
2	*snp-12-590-15828-S-2*	A	G	BB	BB	BB	BB	BB	AB	AA	AB	AA
2	*snp-51-101-82939-S-3*	A	G	AB	AB	BB	AB	AB	AA	AA	AA	AA
2	*snp-89-2-3894-S-3*	A	C	AA	AA	AA	AA	AB	BB	BB	BB	BB
2	*snp-122-75-4354-S-3*	A	G	BB	AB	BB	AB	AB	AA	AA	AA	AA
2	*snp-396-14-12780-S-2*	A	C	BB	BB	BB	BB	BB	AB	AA	AA	AB
2	*snp-21-111-17815-S-1*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
2	*snp-21-212-3930-S-2*	A	G	BB	BB	BB	AB	AB	AA	AA	AA	AA
2	*snp-267-21-1300-S-3*	A	G	AA	AA	AA	AA	AA	BB	BB	AB	AB
2	*snp-72-239-3872-S-3*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
2	*snp-72-205-2923-S-2*	A	G	AA	AA	AA	AA	AA	BB	BB	AB	AB
3	*snp-116-10-4254-S-2*	A	G	AA	AA	AA	AA	AA	AB	BB	AB	BB
3	*snp-17-121-94490-S-1*	A	T	AA	AA	AA	AA	AA	BB	AB	BB	BB
3	*snp-0-92-22510-S-1*	A	G	AA	AA	AA	AA	AA	BB	AB	BB	BB
3	*snp-0-134-12128-S-2*	A	T	BB	BB	BB	BB	BB	AA	AB	AA	AA
3	*snp-0-167-68075-S-1*	C	G	BB	BB	BB	BB	BB	AA	AB	AA	AB
3	*snp-0-194-2943-S-2*	A	G	AB	AB	AB	AB	AB	BB	BB	BB	BB
3	*snp-0-1110-55891-S-3*	A	C	BB	AB	BB	AB	BB	AA	AA	AA	AA
4	*snp-11-433-51531-S-1*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	AB
4	*snp-11-408-32771-S-2*	A	C	AB	AA	AA	AB	AA	BB	BB	BB	BB
4	*snp-11-342-8379-S-1*	A	C	AA	AA	AA	AA	AA	BB	BB	BB	BB
4	*snp-11-315-8528-S-1*	A	G	AA	AA	AA	AA	AA	AB	BB	BB	BB
4	*snp-28-138-3569-S-2*	A	T	AA	AA	AA	AA	AA	BB	AB	AB	AB
4	*snp-28-198-10894-S-3*	A	G	AA	AA	AA	AA	AA	BB	AB	AB	AB
4	*snp-28-234-5414-S-3*	A	G	BB	BB	BB	BB	BB	AA	AB	AB	AB
4	*snp-28-391-75742-S-3*	A	G	BB	AB	AB	AB	AB	AA	AA	AA	AA
4	*snp-3-35-18005-S-1*	A	G	AB	AB	BB	BB	BB	AA	AA	AA	AA
4	*snp-3-177-39024-S-2*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
4	*snp-3-450-40809-S-3*	A	G	AB	AA	AB	AA	AA	BB	BB	BB	BB
4	*snp-3-533-48073-S-2*	A	C	AB	AA	AB	AA	AA	BB	BB	BB	BB
4	*snp-32-262-34508-S-1*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	
4	*snp-32-93-10050-S-3*	C	G	AB	AA	AB	AA	AA	BB	BB	BB	BB
4	*snp-32-88-51840-S-1*	A	G	AB	BB	AB	BB	BB	AA	AA	AA	AA
4	*snp-85-157-3063-S-2*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
4	*snp-31-128-4930-S-2*	A	G	AA	AA	AA	AA	AA	BB	AB	AB	
4	*snp-31-121-188387-S-1*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
5	*snp-45-70-11096-S-2*	A	C	AB	AA	AA	AA	AA	BB	BB	BB	
5	*snp-1-1277-8367-S-1*	A	G	AB	AB	AA	AA	AB	BB	BB	BB	BB
5	*snp-1-1234-21449-S-3*	A	G	AB	AB	AA	AA	AB	BB	BB	BB	BB
5	*snp-1-1081-19826-S-2*	A	G	AB	AB	BB	BB	BB	AA	AA	AA	AA
5	*snp-1-1043-1874-S-3*	A	G	AB	AB	AA	AA	AA	BB	BB	BB	BB
5	*snp-1-1005-6873-S-1*	C	G	AB	AB	AB	AB	AB	AA	AA	AA	
5	*snp-1-387-47907-S-1*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AB
5	*snp-1-311-52989-S-2*	A	G	AB	AA	AB	AA	AA	BB	BB	BB	BB
5	*snp-887-1-10069-S-3*	C	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
6	*snp-22-96-1675-S-1*	A	G	AA	AA	AA	AB	AA	BB	BB	BB	BB
7	*snp-54-40-87969-S-3*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
7	*snp-13-374-46199-S-1*	A	C	AA	AB	AA	AB	AA	BB	BB	BB	BB
7	*snp-13-217-56582-S-3*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
7	*snp-13-214-10867-S-1*	A	G	BB	BB	BB	BB	BB	AA	AA	AB	AA
7	*snp-13-200-129407-S-1*	A	C	AA	AA	AA	AA	AA	BB	BB	AB	BB
9	*snp-25-236-42194-S-1*	A	G	AA	AA	AA	AB	AA	BB	BB	BB	BB
9	*snp-41-278-18296-S-1*	A	G	AA	AB	AA	AA	AA	BB	BB	BB	AB
10	*snp-155-83-3333-S-2*	A	C	BB	BB	BB	BB	BB	AB	AB	AB	AB

10	*snp-70-40-54351-S-2*	A	G	AA	AA	AA	AA	AA	BB	AB	AB	AB
10	*snp-110-94-5270-S-2*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
11	*snp-100-92-1971-S-2*	A	G	BB	BB	BB	AB	AB	AA	AA	AA	AA
11	*snp-92-37-163457-S-3*	A	G	AB	AB	AA	AB	AA	BB	BB	BB	BB
11	*snp-142-64-19874-S-1*	A	C	AA	AA	AA	AA	AA	BB	AB	AB	AB
11	*snp-198-31-4076-S-2*	A	G	BB	AB	BB	BB	BB	AA	AA	AA	AA
11	*snp-107-121-2306-S-1*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
11	*snp-256-1-8355-S-2*	A	G	BB	BB	BB	BB	AB	AA	AA	AA	AA
11	*snp-236-22-4791-S-3*	A	G	AB	AA	AB	AA	AB	BB	BB	BB	BB
11	*snp-156-3-11066-S-3*	A	G	BB	BB	BB	AB	BB	AA	AA	AA	AA
12	*snp-132-21-325303-S-3*	A	G	BB	BB	AB	BB	BB	AA	AA	AA	AA
12	*snp-132-21-335311-S-1*	A	G	AA	AA	AB	AA	AA	BB	BB	BB	BB
12	*snp-16-398-135819-S-2*	A	T	AB	BB	AB	BB	BB	AA	AA	AA	AA
12	*snp-16-64-13646-S-1*	A	G	AB	AA	AA	AA	AB	BB	BB	BB	BB
12	*snp-16-14-84888-S-1*	A	C	AB	AB	AB	BB	BB	AA	AA	AA	AA
12	*snp-49-27-3062-S-1*	A	G	AA	AA	AA	AA	AA	AB	BB	AB	BB
13	*snp-39-143-39018-S-1*	A	G	AA	AB	AB	AB	AB	BB	BB	BB	BB
13	*snp-66-103-27705-S-1*	A	G	AA	AA	AA	AA	AA	BB	AB	BB	BB
13	*snp-66-15-18385-S-2*	A	G	AA	AA	AA	AA	AA	AB	AB	AB	AB
13	*snp-66-14-2353-S-3*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
13	*snp-572-1-2764-S-2*	A	G	AA	AA	AA	AA	AA	AB	AB	BB	BB
14	*snp-30-85-2617-S-3*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
14	*snp-48-169-11772-S-1*	C	G	BB	BB	BB	AB	AB	AA	AA	AA	AA
15	*snp-37-53-28251-S-3*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
15	*snp-37-87-2401-S-3*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
15	*snp-37-104-26184-S-2*	A	G	AA	AA	AA	AA	AA	AB	BB	AB	
15	*snp-37-113-8486-S-1*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
15	*snp-37-140-21234-S-1*	A	C	BB	BB	BB	BB	BB	AA	AB	AA	AA
15	*snp-37-156-6426-S-2*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
15	*snp-37-205-190496-S-3*	A	G	AA	AA	AB	AA	AA	BB	BB	BB	BB
15	*MIF-chr15-8037672*	A	G	AB	AA	AB	AB	AA	BB	BB	BB	BB
16	*snp-368-3-6764-S-1*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
16	*snp-368-9-6157-S-3*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
16	*snp-368-12-1015-S-2*	C	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
16	*snp-368-18-5079-S-3*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
17	*snp-237-54-4101-S-1*	A	G	AB	AB	BB	BB	BB	AA	AA	AA	AA
18	*snp-24-14-6303-S-3*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
18	*snp-24-94-11012-S-3*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
18	*snp-24-191-26961-S-2*	A	G	BB	AB	BB	BB	AB	AA	AA	AA	AA
18	*snp-24-241-10560-S-3*	A	C	AB	AB	BB	BB	BB	AA	AA	AA	AA
19	*snp-19-482-44615-S-3*	C	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
19	*snp-19-480-15842-S-1*	A	C	AA	AA	AA	AA	AA	BB	BB	AB	AB
19	*snp-19-411-14335-S-3*	A	C	BB	BB	BB	BB	BB	AA	AA	AB	AB
19	*snp-19-295-13420-S-3*	A	G	AA	AA	AA	AA	AA	AB	BB	BB	BB
20	*snp-18-218-9766-S-1*	A	G	AA	AA	AA	AA	AA	BB	AB	BB	BB
20	*snp-108-15-32457-S-3*	A	G	AA	AA	AA	AB	AA	BB	BB	BB	BB
20	*snp-108-46-330-S-1*	A	G	AA	AB	AB	AB	AB	BB	BB	BB	BB
21	*snp-52-144-62576-S-1*	A	G	BB	AB	AB	AB	BB	AA	AA	AA	
21	*snp-52-144-82459-S-2*	A	G	BB	AB	AB	AB	BB	AA	AA	AA	
21	*snp-123-54-6694-S-2*	A	G	AB	AB	AB	AB	AA	BB	BB	BB	BB
21	*snp-123-50-16015-S-1*	A	G	AB	AB	AB	AB	BB	AA	AA	AA	AA
21	*snp-123-41-212-S-1*	A	G	AB	AB	AB	AB	BB	AA	AA	AA	AA
24	*snp-50-79-3667-S-1*	A	G	AA	AA	AA	AA	AA	AB	BB	BB	BB
24	*snp-50-156-15077-S-1*	A	C	AA	AA	AA	AA	AA	BB	BB	AB	BB
24	*snp-50-179-22444-S-1*	A	G	AA	AB	AA	AA	AB	BB	BB	BB	BB
24	*snp-50-214-42265-S-2*	A	C	BB	BB	BB	BB	BB	AB	AB	AA	AB

24	*snp-50-240-64873-S-1*	A	G	AA	AA	AA	AA	AA	AB	AB	AB	
24	*snp-119-7-889-S-3*	A	G	BB	AB	BB	BB	AB	AA	AA	AA	AA
24	*snp-119-129-10358-S-2*	A	G	AA	AA	AA	AA	AA	BB	AB	BB	BB
24	*snp-119-145-11226-S-1*	A	C	BB	BB	BB	BB	BB	AA	AA	AA	AA
26	*snp-65-234-365-S-2*	A	G	BB	BB	BB	AB	BB	AA	AA	AA	AA
26	*snp-65-229-2506-S-2*	A	G	AA	AB	AA	AB	AB	BB	BB	BB	BB
26	*snp-65-42-18041-S-1*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
27	*snp-463-23-5865-S-2*	A	G	AB	AB	AB	AB	BB	AA	AA	AA	AA
27	*snp-571-3-9920-S-1*	A	G	AB	AB	AB	AB	AB	AA	AA	AA	AA
28	*snp-149-59-3939-S-3*	A	G	AA	AA	AA	AA	AA	BB	BB	AB	AB
28	*snp-114-27-25093-S-2*	A	C	BB	BB	BB	AB	BB	AA	AA	AA	AA
28	*snp-114-27-1537-S-3*	A	G	AA	AA	AA	AB	AA	BB	BB	BB	BB
Z	*snp-220-54-9693-S-1*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
Z	*snp-78-392-18578-S-2*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
Z	*snp-78-582-5659-S-1*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
Z	*snp-202-52-51925-S-1*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	
Z	*snp-115-435-2924-S-2*	A	T	BB	BB	BB	BB	BB	AA	AA	AA	
Z	*snp-115-277-4143-S-2*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	AB
Z	*snp-135-307-8072-S-2*	A	C	AA	AA	AA	AA	AA	BB	BB	BB	BB
Z	*snp-126-212-2996-S-3*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
Z	*snp-135-2-1768-S-2*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
Z	*snp-154-65-70-S-1*	A	G	AA	AA	AA	AA	AA	BB	BB	BB	BB
Z	*snp-154-6-2743-S-2*	A	C	BB	BB	BB	BB	BB	AA	AA	AA	AA
Z	*snp-234-103-29625-S-2*	A	T	AA	AA	AA	AA	AA	BB	BB	BB	BB
UN[a]	*snp-4211-3-1618-S-1*	A	G	BB	BB	BB	BB	BB	AA	AB	AB	AA
UN	*snp-289-3-2451-S-1*	A	C	AA	AA	AA	AA	AA	BB	BB	BB	
UN	*snp-163-26-6430-S-2*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
UN	*snp-126-116-7856-S-2*	A	G	BB	BB	BB	BB	BB	AA	AA	AA	AA
UN	*snp-2347-3-303-S-1*	A	G	AA	AA	AA	AA	AA	AB	BB	BB	

[a] UN indicates the SNP has not been mapped to one of 39 pairs of chicken chromosomes.

[b] H and L indicate that individuals examined were from lines of chicken divergently selected for high or low antibody titers to sheep red blood cells, respectively.

[c] M and F indicate that individuals examined are male or female, respectively.

Table 6.2 Characteristics of SNPs that were specific for both lines.

Chr	Accession number	Allele[a]		Annotated gene[c]	Gene ID
		H	L		
1	*snp-120-149-1449-S-2*	G	C	*SEMA3A*, sema domain, immunoglobulin domain (Ig) [d]	395825
1	*snp-169-85-6362-S-3*	G	A	*SEMA3C*, sema domain, immunoglobulin domain (Ig) [d]	374090
1	*snp-167-56-17685-S-3*	A	G	*LOC427852*, hypothetical gene, close to *SEMA3E* [d]	427852
1	*snp-6-599-22146-S-2*	C	A	*RCJMB04_20b4*, similar to hypothetical protein, close to interferon-related developmental regulator 1 [d]	417776
1	*snp-7-171-6691-S-2 (VTC05)*[b]	G	C	-	-
1	*snp-7-194-27006-S-1*	G	A	similar to RNA polymerase III subunit 2	418071
1	*snp-29-306-157307-S-2 (VTC07)*[b]	G	A	similar to hypothetical protein, close to toll-like receptor 7 [d]	418636
1	*snp-23-208-36692-S-1*	A	G	similar to Propionyl-Coenzyme A carboxylase, close to integrin, beta-like 1	418774
2	*snp-5-177-14435-S-2*	C	A	similar to hypothetical protein *FLJ22419*, close to *p300/CBP*-associated factor	420651
2	*snp-5-213-47708-S-3*	A	G	-	-
2	*snp-5-236-875-S-1*	G	A	leucine rich repeat containing 3B	420657
2	*snp-5-355-27176-S-1*	A	G	acyl-Coenzyme A dehydrogenase family, member 11, close to *LOC420688*, which is similar to *DnaJ* (*Hsp40*) homolog	420689
2	*snp-5-614-24216-S-3 (VTC04)*[b]	G	A	GLI-Kruppel family member *GLI3*, close to inhibin, beta A	420769
2	*snp-40-25-19645-S-2 (VTC03)*[b]	G	A	-	-
2	*snp-40-160-66815-S-3*	G	A	similar to GDP-mannose 4,6-dehydratase, close to interferon regulatory factor 4 [d]	420890
2	*snp-21-111-17815-S-1*	G	A	-	-
2	*snp-72-239-3872-S-3*	G	A	oxidation resistance 1, close to *LOC420265*, which is similar to dendritic cell-specific transmembrane protein, and close to IL-4 induced gene [d]	420270
4	*snp-11-433-51531-S-1*	A	G	-	-
4	*snp-11-342-8379-S-1*	A	C	-	-
4	*snp-3-177-39024-S-2*	A	G	-	-
4	*snp-32-262-34508-S-1*	G	A	-	-
4	*snp-85-157-3063-S-2 (VTC02)*[b]	A	G	-	-
4	*snp-31-121-188387-S-1*	G	A	-	-
5	*snp-887-1-10069-S-3*	G	C	*RCJMB04_1j22* catalase	423600
7	*snp-54-40-87969-S-3*	A	G	phospholipase A2 receptor 1, close to *LOC424193*, which is similar to lymphocyte antigen 75 precursor [d]	404304
7	*snp-13-217-56582-S-3*	G	A	similar to solute carrier family 35, member F5	424281
10	*snp-110-94-5270-S-2*	A	G	similar to *FLJ21940* protein	415602
11	*snp-107-121-2306-S-1*	A	G	-	-

13	snp-66-14-2353-S-3 (VTC01)[b]	G	A	similar to sequestosome 1, close to LTC4S leukotriene C4 synthase [d]	416284
14	snp-30-85-2617-S-3	G	A	-	-
15	snp-37-53-28251-S-3	G	A	-	-
15	snp-37-87-2401-S-3	A	G	-	-
15	snp-37-113-8486-S-1	A	G	-	-
15	snp-37-156-6426-S-2	G	A	-	-
16	snp-368-3-6764-S-1	A	G	similar to L-amino acid oxidase, close to B-G MHC class IV antigen [d]	417039
16	snp-368-9-6157-S-3	A	G	similar to butyrophilin 1, B locus, close to *LOC417046*, which is similar to IgV-region-like B-G antigen [d]	417045
16	snp-368-12-1015-S-2	A	G	B-lec protein, close to *LOC417046*, which is similar to IgV-region-like B-G antigen [d]	404776
16	snp-368-18-5079-S-3	G	A	similar to class II histocompatibility antigen M beta chain 1, close to *LOC417046*, which is similar to IgV-region-like B-G antigen [d]	417051
18	snp-24-14-6303-S-3	A	G	similar to *DKFZP566O084* protein, close to *LOC417312*, which is similar to *MAP* kinase kinase 4 [d]	417304
18	snp-24-94-11012-S-3	G	A	nudE nuclear distribution gene E homolog like 1, close to *LOC427792*, which is similar to *BCL-6* corepressor isoform c [d]	417317
19	snp-19-482-44615-S-3	C	G	similar to hypothetical protein *FLJ10900*	417478
24	snp-119-145-11226-S-1	C	A	similar to hypothetical protein, close to interleukin 18 [d]	428255
26	snp-65-42-18041-S-1	G	A	progastricsin (pepsinogen C), close to *BTG* family, member 2 [d]	395690
Z	snp-220-54-9693-S-1	A	G	*NFIB* nuclear factor I/B	396209
Z	snp-78-392-18578-S-2	A	G	-	-
Z	snp-78-582-5659-S-1	A	G	similar to 3-phosphatidylinositol kinase 85K chain alpha, close to *LOC431584*, which is similar to lymphocyte antigen 64-like protein [d]	427171
Z	snp-202-52-51925-S-1	A	G	similar to complement component 6 [d]	431585
Z	snp-115-435-2924-S-2	T	A	similar to nicotinamide nucleotide transhydrogenase	427196
Z	snp-135-307-8072-S-2	A	C	similar to transducin-like enhancer protein 1	427450
Z	snp-126-212-2996-S-3	G	A	*ADAMTSL1 ADAMTS*-like 1	431603
Z	snp-135-2-1768-S-2	G	A	similar to Death-associated protein kinase 1	431670
Z	snp-154-65-70-S-1	A	G	similar to GTP-binding protein GL1 alpha chain	431611
Z	snp-154-6-2743-S-2	C	A	-	-
Z	snp-234-103-29625-S-2	A	T	-	-
UN[e]	snp-289-3-2451-S-1	A	C	golgi autoantigen, golgin, subfamily b, macrogolgin 1, close to complement component 7 [d]	426868
UN[e]	snp-163-26-6430-S-2	G	A	RNA binding motif protein 13	426924
UN[e]	snp-126-116-7856-S-2	G	A	-	-

[a] Alleles for each single nucleotide polymorphism (SNP) with one allele in the high antibody (H)-producing individuals examined and with the alternative allele in the low antibody (L)-producing individuals examined, simultaneously.

[b] SNPs located near the candidate markers identified previously (see Chapter 4).

[c] Genes that have been annotated in GenBank.

[d] Candidate genes with known immune response-related functions located within or near (<2Mb) the line-specific SNPs.

[e] UN indicates the SNPs have not been mapped to one of 39 pairs of chicken chromosomes.

Table 6.3 Distributions of SNPs with given characteristics over the respective chicken chromosomes.

Chr/Linkage group	Typed[a]	Informative[b]	Line specific[c]	Candidate gene[d]
1	449	32	8	5
2	321	31	9	2
3	215	7	0	0
4	193	18	6	0
5	147	9	1	0
6	69	1	0	0
7	114	5	2	1
8	86	0	0	0
9	96	2	0	0
10	77	3	1	0
11	64	8	1	0
12	76	6	0	0
13	53	5	1	1
14	51	2	1	0
15	53	8	4	0
16	7	4	4	4
17	47	1	0	0
18	43	4	2	2
19	39	4	1	0
20	51	3	0	0
21	61	5	0	0
22	13	0	0	0
23	9	0	0	0
24	57	8	1	1
25	6	0	0	0
26	35	3	1	1
27	35	2	0	0
28	55	3	0	0
LGE22C19W	1	0	0	0
Z	128	12	11	2
W	8	0	0	0
M	3	0	0	0
UN[e]	69	5	3	1
Total	2733	191	57	20

[a] Single nucleotide polymorphisms (SNPs) successfully typed in the Illumina microarray-based SNP typing assay.

[b] SNPs having at least one allele that was line-specific.

[c] SNPs having one allele in one line and the alternative in another.

[d] SNPs that are line-specific and located within or near immune response related genes.

[e] UN indicates the SNPs have not been mapped to one of 39 pairs of chicken chromosomes.

CHAPTER 7

Microarray-based differential expression analysis of low and high anti-SRBC titers

7.1 Abstract

Global expression profiling has been applied to define the molecular bases of a variety of biological traits through identification of differentially expressed (DE) genes. However, antibody response, as an important trait in both agriculture and biomedicine, has not yet been investigated through global expression profiling. Here, I describe for the first time differentially expressed genes in chickens associated with low or high antibody response based on microarray-based transcription profiling analysis. One hundred and sixty birds from a random-bred population were raised, 145 and 15 of which were challenged and not challenged with SRBC, respectively. In the microarray experiment, 10 non-challenged birds were grouped as N, 10 birds with average anti-SRBC titers were grouped as A, and 15 birds with extremely high or low anti-SRBC titers were grouped as H or L, respectively. Pooled RNA samples made from individual RNA samples for each of the 4 groups were hybridized to Affymetrix chicken GeneChips, with technical replicates. As a result, only 3 genes were identified as differentially expressed between H and L chickens. Gene annotation and classification analysis indicated that they probably were genes influencing chicken anti-SRBC titers. Similarly, 7 genes were differentially expressed between N and A chickens, with one overlapping with genes differentially expressed between H and L chickens. Classification test of these genes suggested that the response of the chicken to intravenous injection of SRBC involved multiple biological processes, including response to stress or other different stimuli, sugar, carbohydrate or protein binding, and cell or soluble fraction, in addition to antibody response. In summary, this preliminary study provides

information useful for identification of genes underlying antibody response to SRBC and sheds some light on the mechanism by which vertebrates respond to pathogen challenge.

Keywords: antibody response, *Gallus gallus*, gene expression, microarray

7.2 Introduction

Microarray technology has emerged as a powerful tool for analyzing mRNA abundance in cells or tissues on a genome-wide scale. Microarray-based transcription profiling analysis has been applied to many types of biological inquiry (Eisen et al., 1998; Yang et al., 2002; Gasch et al., 2000; Chen et al., 2003; Golub et al., 1999; Oberthuer et al., 2006). Identification of candidate genes is an important inquiry. Based on differential expression of genes in cells or tissues under different conditions, candidate genes responsible for a selected phenotype may be identified (Der et al., 1998).

Although different types of gene expression arrays are commercially available, the *in situ* synthesized oligonucleotide arrays, including the Affymetrix GeneChip, are the most comprehensive and complex. This is because Affymetrix GeneChip arrays employ unique array designs in addition to stringent probe selection strategies, including multiple probes for each gene, pairing of a perfect match probe with a mismatch probe, and automated control of the experimental process (Budhraja et al., 2003). These aspects of array design bring about high sensitivity and reproducibility.

Pooling individual samples when carrying out microarray-based transcript profiling analysis is commonly used for reducing experimental costs and sample variation, although it could result in loss of information. Kendziorski et al. (2005) evaluated the utility of pooling and its impact on the efficiency of identifying differentially expressed (DE) genes. They found that inference for most genes was not adversely affected by pooling. They also recommended that pooling be done when fewer than three arrays are used for each experimental condition.

Microarray-based gene expression profiling has been used in several immune-associated studies. For example, Hutton et al. (2004) identified genes preferentially expressed in immune

tissues and cell types. Hess et al. (2004) assessed gene expression changes of naive CD4+ T cells in response to T cell receptor-mediated activation. However, antibody response to heterogeneous erythrocytes (e.g., SRBC) has not been investigated using microarray-based global transcription profiling.

Antibody response is a quantitative trait controlled by multiple genes (Feingold et al., 1976; Biozzi et al., 1979; Siegel and Gross, 1980; Ibanez et al., 1980; Van der Zijpp et al., 1983). It is an important defense mechanism by which vertebrates fight against invasion and infection by pathogens. Selection for antibody response may improve vertebrate disease resistance (Gross et al., 1980). Therefore, identification of genes for antibody response could have important implications in animal production and biomedicine. Heterogeneous erythrocytes often are used as a non-specific antigen for dissecting genetic control of antibody response. Mammalian erythrocytes have a number of membrane proteins on their surface, including glycophorins (Fukuda, 1993; Chasis and Mohandas, 1992), blood group antigens (Lowe, 2001; Cartron et al., 1998), and glycosyl phoshatidylinositol. Because heterogeneous erythrocytes are multi-determinant and strongly immunogenic antigens, it is more likely to identify more of the genes for antibody response than a specific antigen, such as a virus or a bacterium. This is supported by the following evidence. First, background genes, unlinked to immunomodulatory genes at MHC and Igh loci, were shown to contribute to antibody response induced by heterogeneous erythrocytes (Puel et al., 1995, 1996). Second, it was shown that selection for antibody response to heterogeneous erythrocytes was correlated with disease resistance to multiple pathogens (Gross et al., 1980).

Genes or quantitative trait loci for antibody response have been identified by linkage analysis using experimental lines divergently selected for high or low anti-SRBC response in

mouse and chicken (Puel et al., 1995, 1996; Siwek et al.). In addition to major histocompatibility complex (MHC) and *Igh* genes, no other genes were identified by these studies, likely because the regions containing quantitative trait loci are still very large and the exact locations of candidate genes are not defined. Other approaches including microarray-based gene expression profiling may facilitate the identification of these candidate genes.

In this study, I identified genes differentially expressed in spleen between chickens challenged or not challenged with SRBC or between chickens with high and low anti-SRBC titers using microarray-based transcription profiling. This is the first report on the global gene expression profiles of antibody response to SRBC in vertebrates and provides several candidate genes for antibody response.

7.3 Materials and Methods

7.3.1 Animals and tissue collection

A total of 160 1-d-old female chicks were purchased from Fairview Hatchery Company (http://www.fairviewhatchery.com). These birds were from a random-bred egg production strain unselected for antibody production called the Best White Leghorn Egg Layer, which has a medium-size body and lays 300 to 325 white eggs per year. All birds were housed on a wood chip-covered floor. They did not receive vaccinations and were given access to water and feed *ad libitum*. The feed was a pullet starter with 19% crude protein and the required metabolic energy, which met or exceeded National Research Council 1994 requirements. The feed also was supplemented with coccidiostat. When chicks were 45-d old, 15 of them, as non-challenged (N) group, were mock-challenged with 0.1mL of 0.9% saline via the brachial vein, while the rest were administered with 0.1mL of 0.5% suspension of SRBC. Antibody titers to SRBC were

determined 5 days post-injection by hemagglutination assay is described by Siegel and Gross (1980). Titers were expressed as $\log_2$ of the reciprocal of the highest dilution in which there was hemagglutination. Ten birds with average anti-SRBC titers were grouped as A, and 15 birds with extremely high or low anti-SRBC titers were grouped as H or L, respectively. The 4 groups of birds were killed at the next day. Each spleen was immediately collected and put into RnaLater according to the manufacturer's instruction (Qiagen Inc., Valencia, CA). All animal protocols were approved by our Institutional Animal Care and Use Committee.

7.3.2 Total RNA isolation and pooling

Total RNA was extracted from chicken spleen using the RNeasy Mini kit according to the manufacturer's recommended protocol (Qiagen Inc., Valencia, CA). The quality of individual RNA samples was determined using an Agilent BioAnalyzer 2100, and RNA concentration was determined by nano-drop spectrometer. The RNA samples were treated with DNase (Qiagen Inc.) to digest genomic DNA. Equal amounts of total RNA from individual samples then were pooled for each group. within each group were combined. For the N or A groups, individual RNA samples from 10 birds were used. For the H or L groups, individual samples from 7 birds and from the rest were used to make 2 pools for each group. The pooled RNA samples were subjected to assays of quality and quantity.

7.3.3 Microarray assay

GeneChip Chicken Genome Arrays were purchased from Affymetrix (Santa Clara, CA). Each array contained 32,773 transcripts corresponding to over 28,000 chicken genes (https://www.affymetrix.com/support/technical/datasheets/chicken_datasheet.pdf). Ten micrograms of total RNA were labeled and hybridized to the array according to the Affymetrix protocol (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Briefly,

10μL of the total RNA were converted to double-stranded cDNA using the SuperScript Choice System (Invitrogen Life Technologies, Carlsbad, CA) and T_7-$(dT)_{24}$ primer (Affymetrix, Santa Clara, CA). After digestion of double-stranded cDNA, biotin-labeled cRNA was synthesized from the cDNA with the BioArray High Efficiency RNA Transcript Labeling Kit (Affymetrix, Santa Clara, CA). The cRNAs then were purified and fragmented to sizes ranging from 35 to 200 bases by incubation at 94˚C for 35 min. Fifteen μg of the fragmented cRNA was hybridized to a GeneChip at 45˚C with 45 rpm for 16 hours. The GeneChips were washed and stained with streptavidine-phycoerythrin after hybridization, and the signals were further amplified with biotinylated antibody, goat IgG, and another staining with streptavidine-phycoerythrin using Affymetrix Fluidics Station 400 operated by GCOS1.4.0 operation system. Finally, the GeneChips were scanned with an Agilent GeneArray Scanner 3000. Each pooled RNA sample was hybridized to two GeneChips for technical duplication.

7.3.4 Microarray data analysis

A DNA Chip Analyzer (dChip, Li and Wong, 2001a, b) was used for low- and high-level analyses of microarray data. The microarray data initially used by dChip were the *.CEL files created by Affymetrix Microarray Suite Version 5.0 (MAS5.0, Affymetrix), which contained the expression 'signals' acquired from scanning the GeneChips. The low-level analysis included image segmentation, background correction, normalization, model-based expression calculation, and outlier detection. Briefly, the 'invariant set normalization' method (Li and Wong, 2001a) was used to normalize all GeneChips to a baseline chip (LAb2-2), which had the median feature intensity. Expression level then was calculated using a perfect match-mismatch difference model (Li and Wong, 2001b). The model is fit iteratively for each probe set with removal of outliers. The outliers include single, probe, and array outliers (Li and Wong, 2001b). The high-level

analysis included identification of DE genes and gene classification with gene ontology (GO) or protein domain (PD) terms. The DE genes were identified by comparing N with A or L with H data sets. The comparison criteria included fold-change between the group means (not log transformed) exceeding 1.3 using the 'lower 90% confidence bound' or the difference in expression values (not log transformed) between the groups exceeding 300. The median false discovery rate (FDR) was estimated by permutation with n=1000 for multiple-comparison adjustment (Li and Wong, 2001b).

7.3.5 Bioinformatics analysis

Chromosomal locations of differentially expressed genes were determined using their accession numbers in GenBank. Briefly, their accession numbers were used to search against the UniGene database in GenBank for their mRNA sequences, followed by sequence alignments of these sequences against the assembled chicken genome sequence (May 2006 assembly) using the BLAT tool, which is available the UCSC genome bioinformatics site: http://genome.ucsc.edu/cgi-bin/hgBlat?command=start.

7.4 Results

7.4.1 Anti-SRBC titers

The mean anti-SRBC titers ($\log_2$-transformed) for the A, L, H, and all the challenged birds (142 birds except 3 dead) were 7.00, 5.17, 8.37, and 6.78, respectively (Table 7.1). The ratio of H to L in mean anti-SRBC titers (not $\log_2$-transformed) was 9.2.

7.4.2 Quality profile of raw data

Table 7.2 shows the quality profile of the raw data from the microarray experiments. The unnormalized median intensity, the percentage of present calls, the percentage of array outliers,

and the percentage of single outliers ranged from 148 to197, 53.5% to 59%, 0.057% to 1.056%, and 0.088% to 0.327%, respectively.

7.4.3 Differentially expressed genes

Only 3 genes, including *Gga.2929, Gga.4110*, and *Gga.9239*, were identified as differentially expressed between the H and L groups using the comparison criteria (Table 7.3). The standard error of the expression level was small when compared with the expression level of each of these genes (Table 7.3). All of these genes were upregulated in H compared with L. The fold-changes of expression level of the genes range from 1.51 to 2.04 (Table 7.3). However, the fold-changes could range from 1.36 to 3.16 with 90% confidence (Table 7.3). The median FDR, estimated from 1000 times of permutations, was 0 for the three genes, while it was above 25% when more genes were identified as differentially expressed. Two of the three genes are located on chromosomes, 15 and 16 with one not mapped (Table 7.3). Gene annotations indicated that the 3 genes were gamma immunoglobulin heavy (IgG H)-chain 3'-region (HC86) C part, immunoglobulin (Ig) rearranged lambda-chain mRNA variable (V)-region, and a gene similar to MHC Rfp-Y class I alpha chain (Table 7.3). A gene class test with GO and PD terms indicated that *Gga. 9239* was identified as an MHC protein with significant enrichment (p<0.002) (Table 7.5).

Similarly, 7 genes, indicated by 9 probe sets, were identified as differentially expressed between N and A, using the same criteria as for H-L comparison (Table 7.4). However, choosing the criteria for this comparison was not based on the estimated median FDR because N and A groups did not have biological replicates, but only technical replicates, and the median FDR could not be estimated. Interestingly, *Gga.2929* was also identified as differentially expressed between H and L groups; and *Gga.9260* was simultaneously identified by 3 different probe sets,

including *Gga.16267.1.S1_at, Gga.16267.1.S1_s_at*, and *Gga.9260.1.S1_at*. In Affymetrix GeneChips, the same gene may be represented by different probe sets. This is because some probe sets are designed using different segments of the same gene. This study gave such an example. Furthermore, 3 DE genes, including *GgaAffy.20099, GgaAffy.13005,* and *Gga.4942*, were down-regulated with fold-change ranging from -1.87 to -1.43, while the rest were up-regulated with fold change ranging from 1.5 to 1.97 in the A group when compared with the N group (Table 7.4). The estimation of the fold-changes with 90% confidence ranged from 1.33 to 2.49 (Table 7.4). Moreover, the genes are located on chromosomes 1 (2 genes), 5(1), 16(1), and 27(2), with two genes unmapped (Table 7.4). In addition, gene annotations indicated that these genes are heat shock protein 25, heat shock protein 70, lectin-like protein, IgG H-chain 3'-region (HC86) C part, purinergic receptor P2Y, a gene similar to MHC class I alpha chain, and a gene similar to copine VII (Table 7.4). A gene class test of these genes indicated that the response of the chicken to intravenous injection of SRBC involved multiple biological processes, including antibody response (Table 7.6). In particular, *GgaAffy.20099, GgaAffy.13005, Gga.4942, Gga.9260, Gga.4900*, and *Gga.16267* were significantly connected to GO terms that included response to stress or other different stimuli, sugar, carbohydrate or protein binding, and cell or soluble fraction ($p<0.001$).

7.5 Discussion

Candidate genes underlying a quantitative trait can be identified by oligo-nucleotide array-based gene expression analysis (Wayne and McIntyre, 2002; Carr et al., 2007; Scherzer et al., 2007). The number of the candidate genes can be further reduced when combined with QTL mapping (Wayne and McIntyre, 2002; de Buhr et al., 2006; Lai et al., 2007; Joe et al., 2005).

Identifying genes differentially expressed under different conditions or statuses proves a robust approach for microarray-based identification of candidate genes (Carr et al., 2007; Yamashita et al., 2005). In this study, DE genes were identified between the chickens with (A group) or without (N group) SRBC challenge or between the chickens with high (H group) and low (L group) anti-SRBC response using Affymetrix GeneChip arrays. The quality of raw data appeared to be good, which was evidenced by the consistency of the unnormalized median intensities across arrays, the high percentage of present calls, and the low percentages of single and array outliers (Table 7.2). It also was supported by the discovery of the DE genes. Among the DE genes, *Gga.2929* and *Gga.9260* were simultaneously identified by the two comparisons (H-L and A-N) and by 3 different probe sets, respectively, although only a few genes were totally identified (Table 7.3, Table 7.4). For each of them, the direction and level of gene expression were consistent, and biological or technical variations for gene expression level were small within each group (Table 7.3, Table 7.4).

In this study, the A-N comparison identified more DE genes than the H-L comparison (Table 7.3, Table 7.4). The outcome seems reasonable because it is possible for genes to respond to SRBC challenge without influencing anti-SRBC titers. Therefore, it is suggested that the H-L comparison is more efficient in the identification of candidate genes influencing antibody titers than the A-N comparison, although the latter may provide information useful to help us understand the molecular basis of antibody response to SRBC challenge. The A-N comparison indicated that antibody response to SRBC challenge involved multiple biological processes in addition to immune response, including cell or soluble fraction, response to stress or other different stimuli, and sugar, carbohydrate or protein binding (Table 7.6). Interestingly, genes including heat shock protein 25 (*GgaAffy.20099*), heat shock protein 70 (*Gga.4942*), and

purinergic receptor P2Y (*GgaAffy.13005*), which involve response to stress, were down-regulated in response to SRBC challenge (Table 7.4). Moreover, these biological processes may partially agree with the known structure and functions of the spleen (Jeurissen, 1991). The spleen is one of the secondary lymphoid organs and consists of red pulp and white pulp. The network of sinusoids in red pulp is populated with macrophages and numerous erythrocytes and is the site where old red blood cells are destroyed and removed. In comparison, white pulp surrounds the splenic arteries, forming a periarteriolar lymphoid sheath populated mainly by T lymphocytes. Clusters of B lymphocytes in the sheath form primary follicles occupying a more peripheral position (Jeurissen, 1991). Therefore, it is understandable that the functions of spleen are related to the biological processes including immune response, and the cell or soluble fraction.

In this study, IgG H-chain 3'-region (HC86) C part and genes similar to MHC class I were identified as DE genes by both comparisons (H-L and A-N), suggesting that they play roles in response to SRBC challenge and the regulation of anti-SRBC titers (Table 7.3, Table 7.4). The roles of MHC and *Igh* genes in the regulation of anti-SRBC titers were previously described by Puel et al. (1996). However, it is noteworthy that genes similar to MHC class I could be novel candidate genes since the annotations of these genes were based only on sequence similarity. The rest of the DE genes were identified by either of the comparisons. The Ig rearranged lambda-chain mRNA V-region gene was identified only by H-L comparison, suggesting that it may involve regulation of anti-SRBC titers. Previous studies have shown that Ig genes can influence antibody response (Butler, 1998; Rees and Nordskog, 1981). In contrast, genes including heat shock protein 70, purinergic receptor P2Y, heat shock protein 25, lectin-like protein, and a gene similar to copine VII, were only identified by the A-N comparison. The former 2 genes were

previously reported as differentially expressed genes in response to viral infections (Liu et al., 2001; Ruby et al., 2006; Degen et al., 2006).

In this study, the fold-changes (1.51 to 2.04) of expression of the DE genes were much smaller than the ratio (9.2) of H to L in mean anti-SRBC titers. This could be explained by cascade amplification of signaling pathways that involve antibody response to SRBC challenge.

Additionally, the chromosomal locations of all DE genes identified in this study were partially supported by our previous study (Chapter 5), which also indicated that some of candidate genes for anti-SRBC were located on chicken chromosomes 1, 15, and 16. One DE gene, *GgaAffx.13005*, is about 2 megabase from the candidate marker *VTC07*, which was identified using other genomics-based approaches (Chapter 4).

That this study was successful in identification of candidate genes for anti-SRBC response. However, it was limited by the small numbers of individual birds and arrays used in this study and by that examination of only one type of lymphoid organ (spleen). Examining more lymphoid organs (e.g., lymph node and bursa of Fabricius), increasing the number of samples and microarrays, and increasing time points of examination will increase power for identification of DE genes and comprehensive understanding of primary antibody response of vertebrates to SRBC challenge.

Table 7.1 Summary of mean anti-SRBC titers for the chickens challenged with SRBC.

Category[a]	Number of birds	Mean ± SD[b]
A	10	7±0.00
L	15	5.17±0.36
H	15	8.37±0.35
All the challenged	142	6.78±0.95

[a] The birds challenged birds were categorized into average (P), low (L), high (H) groups, and all the challenged White Leghorn birds. N birds are those not challenged with sheep red blood cells (SRBC) in the study A, L and H are those showing average, extremely low and high mean anti-SRBC titers after SRBC challenge, respectively.

[b] The mean anti-SRBC titers has been transformed in $\log_2$. SD: standard deviation.

Table 7.2 Quality profile of raw data from the microarray experiments.

Array ID[a]/*n*[b]	Median Intensity[c] (unnormalized)	P call %[d]	% Array outlier[e]	% Single outlier[f]
N-1/10	153	55.6	1.056	0.327
N-2/10	197	56.3	0.088	0.125
A-1/10	148	57.1	0.864	0.29
A-2/10	196	56.3	0.075	0.115
H1-1/7	155	55.9	0.145	0.142
H1-2/7	158	55.6	0.08	0.123
H2-1/8	180	57	0.182	0.175
H2-2/8	151	59	0.091	0.116
L1-1/7	191	55	0.096	0.118
L1-2/7	163	53.5	0.13	0.141
L2-1/8	188	56.3	0.057	0.093
L2-2/8	172	56.5	0.062	0.088

[a] Each array was hybridized with pooled RNA sample for each of the groups including non-challenge (N), average (A), low (L), and high (H). N birds are those not challenged with sheep red blood cells (SRBC) in the study. A, L and H are those showing average, low and high mean anti-SRBC titers, respectively. Each pooled RNA sample had technical replicates, which are indicated by the last digits (1 or 2) in the array identity (ID) numbers. The pooled RNA sample(s) for each group was made from individual total RNA samples that were extracted from individual chicken spleen. H and L groups each have 2 pooled samples indicated by the first digits (1 or 2) in the array identity (ID) numbers.

[b] The number of individual total RNA samples used for making the pooled samples.

[c] The unnormalized median value of fluorescence intensity across each microarray.

[d] The percentage of probe sets called "Present" in an array. A "Present" is assigned using a Detection *p*-value, which is evaluated against user-definable cut-offs to determine the Detection call. The Detection *p*-value is generated by the Detection algorithm with the use of probe pair intensities (see Data Analysis Fundamentals, http://www.affymetrix.com/index.affx).

[e] The percentage of probe sets called "Array-outlier" in one array. Array outliers refer to an unusual probe response pattern in one array, which is different from the probe response pattern seen in most arrays (http://biosun1.harvard.edu/complab/dchip/manual.htm).

[f] The percentage of "probe pairs" called "Single-outlier" in one array. Single outliers are identified using absolute residuals. If a data point has its absolute residual exceeding both the corresponding array-wise residual threshold and the probe-wise residual threshold, it is called as a single outlier (http://biosun1.harvard.edu/complab/dchip/manual.htm).

Table 7.3 Characteristics of genes that were differentially expressed between high (H) and low (L) antibody-producing chickens.

Probe set ID[a]	Gene	Accession No.[b]	Gene ID	Chromosomal location	L±SE[c]	H±SE[c]	Fold-Change (Range)[d]
Gga.2929.1.S1_at	IgG H-chain 3'-region (HC86) C part	CD737847	*CD737847*	Unknown	3577.6±156.54	5415.91±162.89	1.51 (1.39~1.65)
Gga.4110.7.S1_s_at	Ig rearranged lambda-chain mRNA V-region	BM491664	*Gga.38*	chr15:8,172,126-8,176,698	462.92±97.02	943.94±77.53	2.04 (1.46~3.16)
Gga.9239.1.S1_s_at	similar to MHC Rfp-Y class I alpha chain	AY257166.1	*Gga.9239*	chr16:351,719-368,122	1134.18±149.97	2005.32±195.53	1.77 (1.36~2.35)

[a] Probe set identity on the GeneChip Chicken Genome Arrays (http://www.affymetrix.com/index.affx).

[b] Accession numbers of the differentially expressed genes in GenBank.

[c] Model-based estimate of expression level and standard error (SE) of each transcript in L and H chickens from a commercial White Leghorn population, using dChip software (http://biosun1.harvard.edu/~cli/dchip2006.exe).

[d] Fold-change is the ratio of the model-based estimate of gene expression level in HAb-producing chickens to that in LAb-producing chickens. The range of fold-change is defined by 90% confidence bound.

Table 7.4 Characteristics of genes that were differentially expressed between non-challenge (N) and average (A) anti-SRBC groups of chickens.

Probe set ID[a]	Gene	Accession No.[b]	Gene ID	Chromosomal location	N±SE[c]	A±SE[c]	Fold-Change (Range)[d]
GgaAffx.20099.1.S1_at	heat shock protein 25	AB154518.1	*Gga.985*	chr27:4,486,394-4,487,253	737.61±108.7	395.29±42.02	-1.87 (-1.36~-2.49)
Gga.16267.1.S1_at	similar to class I alpha chain	BU120914	*Gga.9260*	chr16:291,286-354,282	2145.78±85.02	3286.27±136.24	1.53 (1.39~1.68)
Gga.16267.1.S1_s_at	similar to class I alpha chain	BU120914	*Gga.9260*	chr16:291,286-354,282	1373.28±41.71	2184±99.81	1.59 (1.45~1.74)
Gga.4942.1.S1_at	heat shock protein 70	BU118554	*Gga.4942*	chr5:55,409,841-55,412,160	3156.55±102.08	2203±61.72	-1.43 (-1.33~-1.54)
Gga.2929.1.S1_at	IgG H-chain 3'-region (HC86) C part	CD737847	*Gga.2929*	unknown	2507.52±212.1	4939.12±57.81	1.97 (1.73~2.29)
Gga.4900.5.S1_at	Lectin-like protein	BX935608.2	*Gga.37935*	chr1:16,217-21,161	517.21±67.44	953.18±47.56	1.84 (1.49~2.37)
Gga.9260.1.S1_at	similar to class I alpha chain	BU201746	*Gga.9260*	chr16:291,286-354,282	995.82±40.42	1652.43±55.87	1.66 (1.52~1.81)
GgaAffx.13005.1.S1_at	purinergic receptor P2Y, G-protein coupled, 8	AJ720902	*Gga.19558*	chr1:133,187,192-133,213,127	835.2±66.5	473.22±23.84	-1.76 (-1.5~-2.05)
Gga.15701.1.S1_at	similar to Copine VII	CN224397	*Gga.15701*	unknown	718.73±29.95	1075.94±43.04	1.5 (1.36~1.65)

[a] Probe set identity on the GeneChip Chicken Genome Arrays (http://www.affymetrix.com/index.affx).

[b] Accession numbers of the differentially expressed genes in GenBank.

[c] Model-based estimate of expression level and standard error (SE) of each transcript in N and A chickens from a commercial White Leghorn population, using dChip software (http://biosun1.harvard.edu/~cli/dchip2006.exe). N birds are those not challenged with sheep red blood cells (SRBC) in the study. A birds are those with average mean anti-SRBC titers. Here, the SEs were estimated from technical replicates.

[d] Fold-change is the ratio of the model-based estimate of gene expression level in PC chickens to that in NC chickens. The range of fold-change is defined by 90% confidence bound.

Table 7.5 Gene classification test for genes differentially expressed between high (H) and low (L) antibody-producing chickens.

Gene classification (term)[a]	Cluster frequency [b]	Genome frequency of use[c]	*p*-Value[d]	Genes annotated to the term
MHC protein complex (GO)	1:1	17:18040	0.000942	Gga.9239.1.S1_s_at
MHC protein, class I (PD)	1:1	32:18220	0.001756	Gga.9239.1.S1_s_at

[a] Gene classification analysis of the differentially expressed (DE) genes with Gene Ontology (GO) and Protein Domain (PD) terms. The DE genes used here are those differentially expressed between high (H) and low (L) antibody-producing chickens.

[b] The ratio of the number of the DE genes that are annotated to a gene class term to the number of the DE genes that are annotated to all gene class terms found by dChip software.

[c] The ratio of the number of genes that are annotated to a gene class term to the number of genes in the database that are annotated to all gene class terms found by dChip software. The genes used here are retrieved from the annotation CSV file (7/14/06) for the chicken genome, http://www.affymetrix.com/Auth/analysis/downloads/na22/ivt/Chicken.na22.annot.csv.zip.

[d] The statistical significance for the enrichment of the DE genes assessed by gene classification test with either GO or PD term.

Table 7.6 Gene class test of the genes differentially expressed between non-challenge (N) and average anti-SRBC (A) groups of chickens

Gene Classification (term)[a]	Cluster frequency[b]	Genome frequency of use[c]	*p*-Value[d]	Genes annotated to the term
cell fraction (GO)	2:5	68:18040	0.000139	GgaAffx.20099.1.S1_at
				Gga.4942.1.S1_at
immunological synapse (GO)	1:5	17:18040	0.004703	Gga.9260.1.S1_at
sugar binding (GO)	2:5	133:18040	0.000532	Gga.4900.5.S1_at
				Gga.9260.1.S1_at
soluble fraction (GO)	2: 5	19:18040	0.00001	GgaAffx.20099.1.S1_at
				Gga.4942.1.S1_at
protein folding (GO)	2: 5	155:18040	0.000721	GgaAffx.20099.1.S1_at
				Gga.4942.1.S1_at
response to stress (GO)	3: 5	274:18040	0.000034	GgaAffx.20099.1.S1_at
				Gga.4942.1.S1_at
				GgaAffx.13005.1.S1_at
immune response (GO)	1: 5	158:18040	0.043036	Gga.9260.1.S1_at
response to unfolded protein (GO)	2: 5	20:18040	0.000012	GgaAffx.20099.1.S1_at
				Gga.4942.1.S1_at
response to biotic stimulus (GO)	2: 5	58:18040	0.000101	GgaAffx.20099.1.S1_at
				Gga.4942.1.S1_at
response to abiotic stimulus (GO)	2: 5	17:18040	0.000008	GgaAffx.20099.1.S1_at
				Gga.4942.1.S1_at
carbohydrate binding (GO)	2: 5	181:18040	0.000981	Gga.4900.5.S1_at
				Gga.9260.1.S1_at
response to chemical stimulus (GO)	2: 5	73:18040	0.00016	GgaAffx.20099.1.S1_at
				Gga.4942.1.S1_at
response to stimulus (GO)	3: 5	389:18040	0.000096	GgaAffx.20099.1.S1_at
				Gga.4942.1.S1_at
				GgaAffx.13005.1.S1_at
response to protein stimulus (GO)	2: 5	20:18040	0.000012	GgaAffx.20099.1.S1_at
				Gga.4942.1.S1_at
Glycoside hydrolase, family 31 (PD)	1:6	26:18220	0.008533	Gga.9260.1.S1_at
Heat shock protein Hsp70 (PD)	1:6	27:18220	0.00886	Gga.4942.1.S1_at
MHC protein, class I (PD)	2:6	32:18220	0.000045	Gga.16267.1.S1_s_at
				Gga.9260.1.S1_at
Peptidase M1, membrane alanine aminopeptidase (PD)	1:6	29:18220	0.009513	Gga.9260.1.S1_at
Heat shock protein Hsp20 (PD)	1:6	14:18220	0.004602	GgaAffx.20099.1.S1_at
Immunoglobulin-like (PD)	2:6	770:18220	0.023888	Gga.16267.1.S1_s_at
				Gga.9260.1.S1_at
Copine (PD)	1:6	18:18220	0.005914	Gga.15701.1.S1_at

[a] Gene classification analysis of the differentially expressed (DE) genes with Gene Ontology (GO) and Protein Domain (PD) terms. The DE genes are those differentially expressed between (N) and (A) groups of chickens.

[b] The ratio of the number of the DE genes that are annotated to a gene class term to the number of the DE genes that are annotated to all gene class terms found by dChip software.

[c] The ratio of the number of genes that are annotated to a gene class term to the number of genes in the database that are annotated to all gene class terms found by dChip software. The genes used here are retrieved from the annotation CSV file (7/14/06) for the chicken genome, http://www.affymetrix.com/Auth/analysis/downloads/na22/ivt/Chicken.na22.annot.csv.zip.

[d] The statistical significance for the enrichment of the DE genes assessed by gene classification test with either GO or PD term

OVERALL SUMMARY

In this dissertation research, multiple resources have been developed for defining the molecular basis of antibody response in chickens. These resources include the resource population that consists of the divergently selected lines and crosses derived from them, candidate markers identified by RAPD and SCAR analyses, line-specific SNPs identified by microarray-based SNP analysis, and candidate genes identified by microarray-based gene expression analysis.

In the resource population, large phenotypic divergence in the selected lines suggested that selection has caused differentiation of allele frequencies at QTLs associated with anti-SRBC response in the lines. This inference was initially supported by the analysis of 3 candidate markers that were associated with antibody titers to *S. enterica*, and was further supported by the RAPD and SCAR analyses and microarray-based SNP analysis.

Using the divergently selected lines, 6 out of 555 random primers were revealed by RAPD analysis to amplify line-specific RAPD fragments, and 57 line-specific SNPs identified by microarray-based SNP analysis were primarily on chicken chromosomes 1 (8 SNPs), 2 (9), 4 (6), 15 (4), 16 (4) and Z (11) were. Seven SCAR markers converted from line-specific RAPD fragments, which were amplified by 4 random primers, were evaluated for their associations with anti-SRBC response in both the divergently selected lines and the backcross population using SNPs identified in the markers and flanking regions. The allele frequency of each of the 7 SCAR markers were significantly different ($p<0.0025$) between the parental lines, and two of which were also significantly ($p<0.05$) associated with anti-SRBC in the backcross population. Their chromosomal locations were genetically and physically mapped and used for identifying immune response-related genes. Eighteen immune response-related genes were near the SCAR markers

(<2.5 Mb). Moreover, 6 of the SCAR markers were shown to be near (<2 Mb) the line-specific SNPs that were identified by the microarray-based SNP analysis. In addition, PCR-RFLP methods were developed for genotyping one informative SNP within each of 6 SCAR markers. Using these methods, the allele frequency distributions of the informative SNPs were evaluated in other 7 chicken populations that were not directly selected for antibody response. The results from the PCR-RFLP analysis suggested that the significant difference in allele frequency of each SCAR marker between the selected lines was probably not due to genetic drift.

In the microarray-based gene expression analysis, 3 genes were differentially expressed between chickens identified as high and low anti-SRBC response, and 7 differentially expressed genes, with *Gga.2929* overlapping with the former, were identified between chickens challenged and non-challenged with SRBC. Among these genes, *Gga.Affy.13005* (Purinergic receptor P2Y) was close (about 2 Mb) to the SCAR marker *VTC07* that was identified by RAPD and SCAR analyses. The chromosomal locations of the differentially expressed genes were on chromosomes 1, 5, 15, 16 and 27, which was partially consistent with the microarray-based SNP analysis.

This dissertation research is resource-driven, although it is based on the hypothesis that difference among chickens for anti-SRBC titers is polygenic. Candidate markers including SNPs and differentially expressed genes identified in the dissertation research may provide a resource for future study of the molecular basis of antibody response to pathogens. However, the dissertation research was limited in some aspects. First, the number of individuals from backcross population used for the SCAR-QTL analysis was small. Second, the number of chickens and the SNP typing capacity of the Illumina microrray (about 1 SNP per 400 kb of chicken genome) were limited in the microarray-based SNP-QTL analysis. Last, only one type of

lymphoid organ was examined, the number of samples analyzed was small, and the result has not yet confirmed by quantitative RT-PCR, in the microarray-based expression QTL analysis.

In the future, the dissertation research could be further deepened and enhanced by the following work. First, QTLs underlying anti-SRBC response could be identified through fine-mapping approaches. Second, quantitative RT-PCR could be used to validate the expression levels of the differentially expressed genes identified by microarray-based expression QTL analysis. Third, more differentially expressed genes associated with anti-SRBC response could be identified by increasing sample size and examining additional lymphoid organs (e.g., bursa of Fabricius and lymphoid nodule). Last, candidate markers could be validated in other chicken populations.

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VITA

Tuoyu Geng was born in 1967 in Yangzhou, Jiangsu Province, China. He received his B.S. degree in Animal Science in 1989 and M.S. degree in Animal breeding and Genetics in 1992 from Yangzhou University. He then joined the Institute of Poultry Science, Chinese Academy of Agriculture Science as a research assistant. He was appointed as research associate in 1993, research assistant professor in 1995, and research associate professor in 2001. He moved to Blacksburg, VA and worked in Dr. Ed Smith's Lab as a visiting scholar in 2002. He was appointed as research associate in the next year. In the fall of 2005, he began his Ph.D. study under the direction of Dr. Ed Smith in the Department of Animal and Poultry Sciences, Virginia Tech. He earned a Ph.D. in Spring 2007. He is a member of Sigma Xi, the Scientific Research Society.