IDENTIFICATION OF MHC HAPLOTYPE RELATED WITH AVIAN INFLUENZA VIRUS DISEASE IN THAI INDIGENOUS CHICKEN

INTRODUCION

The Thai indigenous chicken (*Gallus gallus domesticus*) originated from the Red Jungle Fowl. They were kept in small flocks for home consumption. They had been selected by natural selection. Animal resisting to infectious disease could survive and were reproductive, thus the result was the origin of the Thai indigenous chicken. In the rural areas of Thailand, farmers raise indigenous chicken to be food, pets, fighting chicken, and alarm clock. The flavor of Thai indigenous chicken meat was delicious and appropriate to Thai cookings. Accordingly, farmers raised Thai indigenous chicken for their home consumption and supplementary income.

During recent times, poultry industry was confronting increasing problems due to the outbreak of ongoing more virulent forms of pathogenic viruses, which needed more effective vaccines to prevent disease. However, it was confirming that vaccination ineffectually againsted very virulent strains and those which could affect human. Chicken suffered from a number of diseases, some of which were fatal, all of which affected the growth, production and health of the birds. Consequently, disease resistance was an important economical consideration to poultry industry. The economic importance of disease to chicken production was difficult to estimate as costs are not only direct (mortality and morbidity) but indirect as well (vaccinations, chemotherapy, and eradication programs). During November 2003 to March 2004 in many country in Asia, there were outbreaks of H5N1 avian influenza virus (AIV), causing of death of infected patients and devastated poultry industry. Avian influenza outbreaks could be difficult to control and often caused major economic impacts for poultry farmers in affected countries, since mortality rates were high and infected fowl generally might be destroyed in order to prevent the spread of the disease. As a result of ongoing outbreak in Asia, FAO estimated that around 20-25 million birds had been destroyed (FAO, 2004). For this reason, the local economies of both commercial poultry operations and smallholders were devastated, especially in Thailand, where the industry was heavily reliant on trade. In 2003, poultry exports from Thailand accounted for nearly 7 percent of global poultry meat trade, with an export value of approximately US$1 billion (FAO, 2004). Avian influenza virus disease outbreak severely decreased frozen poultry meat exportation. The most importance of this outbreak, it caused of death of infected patients in Thailand and Vietnam (FAO, 2004). Thus, disease resistance was an important economical consideration to poultry industry. During this outbreak, some Thai indigenous chicken survived, suggested they were AIV resistance. This situation indicated that disease resistant traits can be improved genetic selection of genetic markers related to these traits.

The AIV outbreak also might destroy Thai domestic supplies as well as exports of this important food commodity. An interesting alternative approach to standard disease control methods would be selective breeding to increase disease

resistance in livestock. Genetic resistance to disease involved many body's defense systems and their interactions and was extremely complex. Disease resistance research had included measurement of genetic control of disease losses, estimation of heritabilities, and characterization of breed or strain differences. New opportunities to improve our understanding of the genetic nature of disease resistance now occur through the recent advances in molecular biology, gene mapping, and immunology and make selection for disease resistance possible in the future. However, genetic considerations included with testing and selection for disease resistance and improved immune responsiveness would need knowledge of the genetic correlations among disease resistance and immune responsiveness and production traits. Consequently, it would be useful to know the identity of the gene or genes affecting the resistance traits for each disease, as with this detailed information it would be possible to directly select for the required trait. The current focus was on the genetic makeup of the chicken, specifically the major histocompatibility gene complex (MHC) lied on chromosome 16 of chicken. Genes of MHC would possibly affect how chicken responded to diseases and vaccines. It had a highly polymorphic genetic system. Its products worked as a central role in immune responses by presenting antigenic peptides to T lymphocytes. Due to this involvement in immune responses, the MHC had a large influence on resistance or susceptibility to disease. This promoted the identification of specific chicken MHC haplotypes for disease resistance. Knowledge of the gene might also suggest improved pharmaceutical or immunological therapies.

OBJRCTIVES

1. To identify MHC haplotypes of Thai indiginious chicken by using LEI0258 microsatellite marker and SSCP method

2. To study genetic diversity of MHC genes of Thai indiginious chicken

3. To study influences of MHC haplotypes on Avian Influenza Virus Disease resistant traits in Thai indiginious chicken

SCOPE OF RESEARCH

This research was separated into two part

1. Identification of MHC class I and II haplotypes of Thai indiginious chickens raised in Livestock Research and Breeding Center of DLD (reference population). They were composed of Leung-Hahng-Kow (LHK), Pradoo-Hahng-Dam (PHD), Chee (CH), Nok-Dang (ND), and Kau-Laun (KL). They were raised at Kabinburi Livestock Research and Breeding Center (KB), Tabkwang Livestock Research and Breeding Center (TK), Thapra Livestock Research and Breeding Center (TP), and Surathani Livestock Research and Breeding Center (ST).

2. Identification of MHC class I and II haplotypes of Thai indiginious chicken of small holder farms in the area of AIV outbreaks (death and survivor) in central part of Thailand, these haplotype were estimated for influences of MHC haplotypes on AIV traits.

LITERATURE REVIEWS

Thai indigenous chicken

It was expected that Thai indigenous chicken (*Gallus gallus domesticus*) originated from the Red Jungle Fowl. They were kept in small flocks for home consumption and had a large diversity between many lines in this breed. They were separated into many strains. Five of them: Leung-Hahng-Kow (LHK), Pra-Doo-Hahng-Dam (PHD), Nok-Dang (ND), Chee (CH), and Kau-Laun (KL) were kept for breeding goal in the research and breeding center of Department of Livestock Development (Division of Animal Husbandry, 2003).

Leung-Hahng-Kow (LHK)

The LHK chicken was one major strain of Thai indigenous chicken. It originated from Oo chicken. Some of them were raised as fighting chicken in Sookohthai kingdom era. After harvesting time, farmers brought LHK chicken to fighting sport. Later, this sport was popular in nobleman. It was developed as the king's sport in Sookothai and Ayoottaya kingdom era such as King Naraysuan, King Surah, and King Taaksin. The most popular of LHK chicken was LHK chicken at Ban-Krang and Ban-Hua-Tay district in Pissanoolok province. In former times, King Naraysuan as an unofficial captive of Burma. He brought LHK chicken to Burma to fight with chicken of Burma Crown Prince. The LHK chicken won chicken of Burma Crown Prince (Division of Animal Husbandry, 2003).

Average mature weight of male and female LHK chicken were 3.5 and 2.0 kgs. The LHK chicken was clever and swift. They had big muscle such as breast, shoulder, and legs. Beak color was yellowish-white. Nose color was light yellow. They had yellow eyes and red pea comb. Wattle color was red. Feather color of wing was black and mixed with dark yellow and white. Tail feathers were very long with white color. They had light yellow leg scales (Division of Animal Husbandry, 2003) (Figure 1).

Male **Female**

Figure 1 Leung-Hahng-Kow chicken
Source: Division of Animal Husbandry (2003)

Pra-Doo-Hahng-Dam (PHD)

The PHD chicken was one of other major strain of Thai indigenous chicken. It originated from Oo chicken similarly LHK chicken. Some of them were developed side by side with LHK chicken as fighting chicken in Sookohthai kingdom era. It was popular and developed as the king's sport in Ayoottaya kingdom era such as King Naraysuan, King Surah, and King Taaksin. The most popular of PHD chicken was PHD chicken in Soopunburi, Singhburi, Ahngthong, Ayoottaya, Chacherngsow, and Bangkok provinces. These areas were the origin of supreme PHD chicken. It was very popular in King Aygahthosarot in Ayoottaya kingdom era. King Aygahthosarot brought it from Soopunburi province to fight with chicken of official. It often won other chicken. The PHD originated of other Thai indigenous chicken (Division of Animal Husbandry, 2003).

Average mature weight of male and female PHD chicken were 3.0 and 2.0 kgs. They had big muscle. Beak color was dark yellow or brown. Nose color was dark yellow. They had dark zingiber yellow eyes and red pea comb. Wattle color was red. Feather color was greenish black. Tail feathers were very long with black color. They had dark yellow leg scales (Division of Animal Husbandry, 2003) (Figure 2).

Male

Female

Figure 2 Pra-Doo-Hahng-Dam chicken
Source: Division of Animal Husbandry (2003)

Nok-Dang (ND)

The ND chicken was one of other major strain of Thai indigenous chicken and fighting chicken. It was a pure breed chicken in central, southern, and northern part of Thailand. It was popular in middle period of Ayoottaya kingdom era. It was majority raised in Karnjanaburi, Pissanoolok, Nakornsawan, Ayoottaya, and Phattalung provinces (Division of Animal Husbandry, 2003).

They had yellowish red beak. They had red eyes and red pea comb. Wattle color was red. Feather color was red. Tail feathers were similar to horse tail. They had yellowish red leg scales (Division of Animal Husbandry, 2003) (Figure 3).

Male

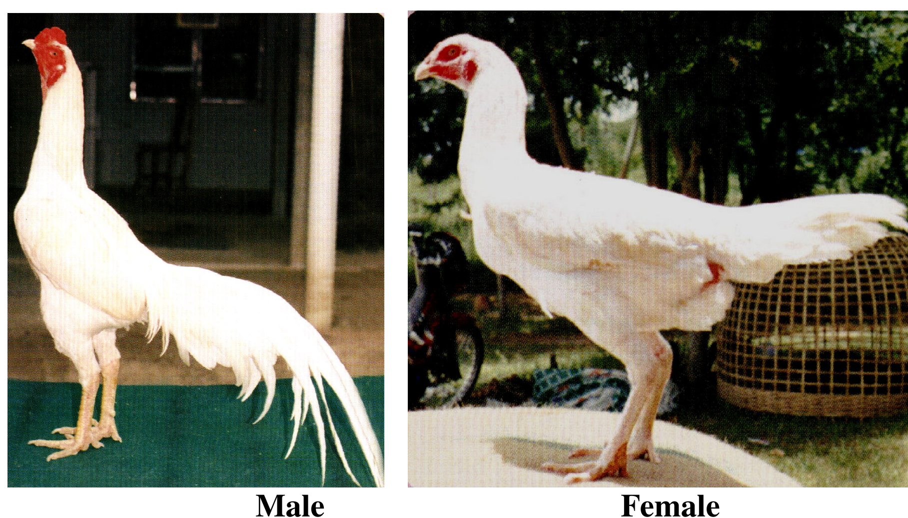
Female

Figure 3 Nok-Dang chicken
Source: Division of Animal Husbandry (2003)

Chee (CH)

The CH chicken had white feathers. It originated from Leung -Hahng-Kow chicken. It was chicken in central, and northern part of Thailand. It was majority raised in Pissanoolok, Kumpangpet, Sookohthai, Ayoottaya, Ahngthong, Singhburi, and Pechburi provinces (Division of Animal Husbandry, 2003).

Average mature weight of male and female CH chicken were 3.0 and 2.0 kgs. They had yellowish white beak. They had light yellow eyes and red pea comb. Wattle color was red. Feather color was white. Tail feathers were similar to horse tail. They had yellowish white leg scales (Division of Animal Husbandry, 2003) (Figure 4).



Male **Female**

Figure 4 Chee chicken
Source: Division of Animal Husbandry (2003)

Kau-Laun (KL)

The KL chicken was one of Thai indigenous chicken in southern part of Thailand, especially, in Patthalung province. It was not revealed about its origin. Average weights of male and female KL chicken at 6 months were 1.8 and 1.6 kgs. KL chicken had high percentage of breast part (Division of Animal Husbandry, 2003) (Figure 5). The KL chicken lacked of feathers in the neck region. The unfeathered area extended to about half way down the neck; an area with sparse feathering. This characteristic was an economic importance of heat tolerance, which was a special advantage for a tropical country like Thailand. They could grow well under bad environment, poor housing, management and nutrition with variable temperature and relative humidity. Because of their fewer feathers, they required less protein and this might result in a reduced incidence of feather peaking and cannibalism. With less feather cover, the skin of these birds could receive more solar radiation, which may facilitate greater vitamin D3 synthesis and in turn, contributed to the better egg shell quality and better health.

Figure 5 Kau-Laun chicken
Source: Division of Animal Husbandy (2003)

Major Histocompatibility Complexes

Major Histocompatibility Complex (MHC) was investigated as self antigen, different from non-self antigen. This special complex was needed to help the immune system differentiate between which cells were parts of the body, and which cells were foreign. MHC was glycoprotein on cell surface encoding by MHC genes. These genes were discovered by the group of scientists who won the Nobel Prize in Medicine in 1980. The discovering of these genes helped making human organ transplants possible (Benacerraf *et al.,* 1980). MHC was important group of molecules that determined whether tissues were compatible. MHC was critical in the functioning of the immune system. System of MHC was a highly polymorphic genetics and has been shown as a group of genes, seem to be familiarly associated with both disease resistance and immune responsiveness. All higher life forms are known to possess a MHC that coded for the predominant cell surface proteins on cells and tissues of each individual species. These antigens were markers of "self" and were unique for animals other than identical twins or clones. These "self" molecules or MHC proteins selected the virus containing cell for destruction by presenting antigenic peptides to T lymphocytes (Abbas *et al.,* 2000). Due to this association in immune responses, the MHC had a large influence on resistance or susceptibility to disease.

Pazderka *et al.* (1975) had studied in chicken MHC, reported that it was the ability of leukocytes to give strong graft rejection. There was some proof for different B types having different competence in graft vs. host responses (GVHR), and this was confirmed as a mechanism for resistance to Marek's disease; i.e., less proliferation would decrease the chance of lymphoma development, but might also lead to a general decrease in T cell responses. Additional proof that B displayed the chicken MHC was given by the identification of B as the graft versus host (GVH) splenomegaly locus, the major GVH chorioantoic membrane (CAM) pock locus and the mixed lymphocyte reaction (MLR) locus. These genes affect the immune response, histocompatability reactions, and response to disease (Jaffe and McDermid, 1962; Miggiano *et al.*, 1974).

Chicken chromosome 16 carried MHC (B complex and Rfp-Y complex) (Pink *et al*., 1977; Guillemot *et al.*, 1988; Bumstead and Palyga, 1992). The Rfp-Y complex closed to nucleolar organiser region (NOR) (Dominguez *et al.*, 1991; Miller *et al.*, 1994b) and was accordingly of considerable immunological interest. The NOR carrying the 5.8S, 18S, and 28S ribosomal RNA genes (rDNA) for protein synthesis. Chicken only had one copy of rDNA containing of a cluster of 14540 kb repeats, which represented about 0.5% of the chicken genome (Wain *et al*., 1997). The NOR was shown to exist on the same chromosome as the chicken MHC by trisomy mapping of a bird expressing B^6, B^{13}, and B^{15} antigens (Bloom and Bacon, 1985). Estimates of the size of the microchromosome range from 8 Mb upwards; however, the NOR occupied a large portion of the chromosome, possibly as much as 6 Mb. The MHC region was therefore perhaps 2 Mb or 0.17% of the genome.

van der Zijpp (1983) had studied the B complex and shown that they were associated with both immune response and disease resistance. More particularly, the B complex had been shown to be involved with immune response to unnatural antigens, bovine serum albumin, *Salmonella pullorum* bacterium, total IgG levels, and cell mediated responses. Resistance to Marek's disease, Rous sarcoma virus, fowl cholera, and lymphoid leukosis viruses had also been demonstrated to be associated with the chicken MHC (Lamont, 1989).

There were three classes of protein molecules, class I, class II and class III, were encoded by the MHC in mammalians (Abbas *et al.*, 2000). Class I molecules were generally direct cellular immune responses and class II molecules were associated in stimulating both cellular and antibody responses. Both classes of MHC molecules were highly polymorphic, differing even between closely related animals, and these differences modify the affinity of the MHC proteins for different peptide antigens. In inbred laboratory animals, it had been relatively easy to demonstrate that difference in reactivity to simple antigens is linked to MHC polymorphism. Class III molecules show only limited polymorphism. The function of the MHC genes was now generally known. Quddus *et al.* (1986) reported that the class I molecules controled the specific function of T cytolytic lymphocytes, acted as restraining elements in T cell recognition of virally infected target cells and, consequently, were essential to generate an immune response. The class II genes controlled the interaction of T cells, B cells, and macrophages in the production of the humoral immune response and participate, as well, in expressions of cellular immunity. The class III genes were signifiably associated with the complement cascade, which ended with the lysis of the cell or virus particle to which antibody had bound.

Li *et al.* (1997) showed that the chicken B complex included several linked multigene families of highly polymorphic genes. It was afterward discovered to contain three loci, BF, BL and BG (Pink *et al*., 1977), producing class I, II and IV antigens respectively (Nordskog *et al.*, 1987). The class I region (BF) had been expandable studied because of its involvement with susceptibility to Marek's disease (MD) which was the most prominent naturally appearing disease of chicken. Class I antigens were indicated on the surface of all nucleated cells (Ewert *et al.*, 1980), as a single transmembrane polypeptide chain folded into three extracellular domains

involved non covalently with β_2 microglobulin (Figure 6). Class I molecules held and presented forein antigens for recognition by CD8 cytotoxic T lymphocytes if the cell was infected by a virus or other microbe (Abbas *et al.*, 2000). This region was homologous to HLA-A, B, and C genes in man, and homologous to the murine K and D loci (Simonsen *et al.*, 1980). MHC class II molecules were the billboards of the immune system. Peptides derived from foreign proteins were inserted into MHC's binding groove and displayed on the surface of antigen presenting cells (Ewert *et al.*, 1980). These peptides were then recognized by CD4 helper T lymphocytes so that the immune system was active to the presence of foreign material (Pharr *et al.*, 1993; Chen *et al.*, 1997). Class II antigens were indicated on immunological cells as heterodimers with two non variable immunoglobulin like domains near the membrane and two variable domains, furthest from the cell. This region is homologous to the HLA-D genes in man. Class IV antigens were expressed on the cell surface of erythrocytes and are unique to avian species but were of unknown relationship to the H2 complex and HLA loci (Simonsen *et al.*, 1980). They contained of two glycoproteins which are highly polymorphic, but of unknown function. However, they were distantly related to butyrophilin and myelin oligodendrocyte glycoprotein (Kaufman *et al.,* 1995). BG was apparently restricted to erythrocytes and is of unknown relationship to the H2 complex and HLA loci (Simonsen *et al.,* 1980).

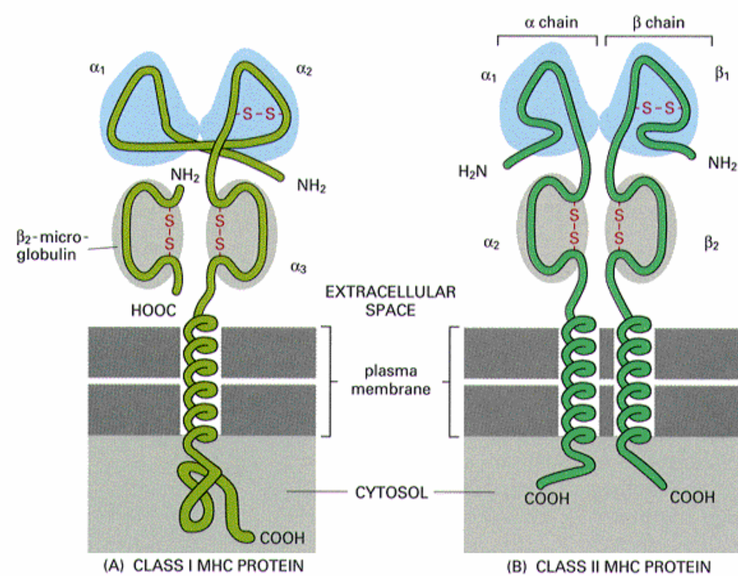


Figure 6 MHC classI molecules (in mammalian) are composed of a polymorphic chain noncovalently attached to the nonpolymorphic microglobulin. MHC classII molecules (in mammalian) are composed of a polymorphic chain noncovalently attached to a polymorphic chain.

Source: Abbas *et al*. (2000)

Each class of MHC genes was a latent candidate for a role in disease resistance. Uni *et al*. (1993) showed that the class IV genes related to early antibody

production, and this connection showed the potential of expected breeding not only by immunological phenotype but also by genotype. The MHC genes showed involvement with response to diseases as miscellaneous as virally induced neoplasia, bacterial, parasitic and autoimmune diseases.

Hala *et al*. (1976) showed that the chicken B complex was frequent recombination appeared between BF and BG regions, though none had been detected between BF and BL. The knowledge that frequent recombination occurred between the BF and BG regions was critical (Briles *et al*., 1982), since the BF region was thought to control resistance, and a particular BF region might not always be involved with the same BG region. Recombination chances separating the BG region and the classical BF/BL region had been reported in experimental matings, with a frequency of about 1/2000 (Koch *et al*., 1983). At least part of the difference in serological conformations was due to glycoproteins encoded in the BF IV locus. There is intra allelic recombination or exon shuffling involving the 2 domain (Hunt *et al*., 1994).

There were two class I genes: BF1 and BF2 were placed on BF/BL region within part of the B complex (Kaufman *et al*., 1999, Miller *et al*., 2004). The TAP2 (Transporter associated with antigen processing 2) gene lied between these two genes (Bumstead *et al*., 1994). In the same BF/BL region were also located one of many BG genes and the gene encoding the β subunit of a GTP binding protein (Guillemot and Auffray, 1989). Moreover, the nucleotide sequences of the variable (β1) exon of BLβII family genes from 17 standard Leghorn haplotypes were known (Zoorob *et al*., 1993), as were sequences of six BF alleles (Guillemot *et al.,* 1988; Kaufman *et al*., 1992; Hunt *et al*., 1994; Pharr *et al*., 1994; Fulton *et al*., 1995). Li *et al*. (1997) reported that sequences of α1/α2 domains from additional BF alleles/isotypes could be found in the GenEMBL database. However, the BF sequence attained from the B^{A4v} haplotype was different from all published BF sequences (Guillemot *et al*., 1988; Kaufman *et al*., 1992; Hunt *et al*., 1994; Pharr *et al*., 1994; Fulton *et al*., 1995) and from additional BF sequences in the GenEMBL database (Li *et al*., 1997).

The class II genes were interspersed with the class I genes along a relatively small region of chromosome 16, the BF/BL region, represented in a cosmid cluster of 130 kb. There were at least 5 class II *β* genes in total, two of which were known to be expressed and lied within the BF/BL region about 8 kb apart. The BLβ genes were transcribed specifically in tissues containing cells of the B lymphocyte and myeloid lineages that expressed the BL antigens. Bourlet *et al*. (1988) showed that exons encoding the β1, β2 and transmembrane domains of a BLβ chain were identified, and displayed 63, 66 and 62% similarity with a human HLA-DQ *β* sequence. This was the first isolation of an MHC class II gene other than in mammals. In chicken, the intron/exon structure for class II was similar to that in mammals, but with much smaller introns. The total gene sizes were less than 2 kb in comparison to 8-20 kb in man (Guillemot and Auffray, 1989). At present, only one class II α gene had been found (Kaufman *et al*., 1993), about 5 cM away from the BF/BL region.

Jacob *et al*. (2000) reported that the BLβII and BLβVI genes are two lineages of BLβ genes and presented that they all be named BLβ genes. The structural

organization and expression of the class II β chain genes in the BF/BL region was similar to that of fowl class I (BF) genes, one functional result of which is differential resistance to disease and response to vaccines. The functional BL βII genes were compatible to the genomic organisation and tertiary structure of class II β molecules were remarkably conserved between birds and mammals (Zoorob *et al.*, 1990).

Pharr *et al.* (1993) found that the sequences of subclones, derived from the BLβI and BLβII genes of the fowl MHC, showed that the genes were identical for most lines with the same MHC haplotype, but for one haplotype (B^{19}), there were variation between the class II genes even within lines that appeared to be the result of micro recombination (gene conversion). Comparison of the nucleotide and predicted amino acid sequences of the 2 subclones with other class II chain sequences showed that the B^6 chain genes were evolutionarily related to the class II chain genes from fowls with other MHC haplotypes, and to class II chain genes from other species. Analysis of Southern blots of B^6 fowl DNA, as well as the isolation of the 3 chain genes, suggested that fowls with the B^6 haplotype possessed at least 3 MHC class II chain genes (Xu *et al.,* 1989). The class II genes of birds had until now only been molecularly identified in the domestic chicken. The pheasant genes were highly variable, although one of the amplified sequences was found in two different haplotypes. The most polymorphic positions (Guillemot *et al.,* 1988) were taken together not identical in any of the predicted protein sequences, but all except one of the motifs had already been found in the domestic chicken. Structurally important characteristics in mammalian class II genes were generally conserved also in the pheasant sequences, but the loss of a potential salt bridge component in several sequences might suggest a slightly different structure of the adjoining parts of the peptide binding groove. The pheasant genes were most closely related to the so called BLII family in the chicken, indicating that this represented a major line of development among avian class II genes.

Frangoulis *et al.* (1999) found that the chicken MHC class I α1 and α2 domain genes and MHC class II β1 and β2 domain genes (CIIB) were investigated as candidate genes for antibody response. MHC genes or others linked in the MHC complex played very important roles in genetic control of antibody response kinetics in chicken. The Tapasin (TAP binding or associated protein) molecule played a role in the group of major histocompatibility complex (MHC) class I molecules in the endoplasmic reticulum, by mediating the interaction of class I β2 microglobulin dimers with TAP. Like its human homologue, fowl Tapasin contained 8 exons, but had a significantly reduced intron size compared with the human gene. Fowl Tapasin coded for a transmembrane protein with a probable endoplasmic reticulum retention signal. Exons IV and V, and possibly exon III, coded for devide domains that were related to the immunoglobulin (Ig) superfamily. The fowl Tapasin gene was localized to the centromeric end of the MHC (B complex), between the class II BLβ genes.

Sung *et al.* (1993) suggested that the structure of the BL molecules of the fowl haplotype was similar, but not identical, to that of the mammals. Comparisons of the nucleotide and predicted amino acid sequence of the MHC class II β chain genes of fowls and mammals indidated species specific differences, the structurally

homologous domains had been under different selective pressures during evolution (Kaufman *et al*., 1992).

Wain (1997) reported that there were many BG genes, a number of which were transcribed. Nevertheless, their positions on the chromosome were not as yet defined, although one clone maps to the same linkage position as TAP2. Another gene mapping to this linkage position was the chicken homologue of G9a (BAT8), which was linked to the class III genes in man (Spike and Lamont, 1995). One of the few identified class III genes, C4, also placed within this region (Wain, 1997). There were reports that BG alleles occurred in different combinations with BF and BL alleles in lines of chicken other than leghorns (Simonsen *et al.*, 1982).

Kaufman *et al.* (1999) found that the chicken major histocompatibility complex (MHC) had been shown to contain only 19 genes within a 92 kb region of the B locus. It was consequently around 20 times smaller than the human MHC, yet its genes had analogues in the human. The human MHC region, on the other hand, sprawled over nearly 4 million bases and comprised around 180 functional genes, about 40% of which were associated with the workings of the immune system. The minimal MHC of the chicken was likely to be more representative of the simple MHC region. This recommended that a minimal essential set of MHC had been defined and conserved for 300 million years of dividing between birds and mammals. The avian and mammalian MHC were established differently, with the class III region genes placed outside the class II and class I region genes. The nonappearance of proteasome genes in the chicken MHC was unexpected and might explain unusual peptide binding specificities of chicken class I molecules. The presence of putative natural killer receptor genes was unprecedented and might explain the importance of the B locus in the response to the herpesvirus that causes Marek's disease.

Briles *et al*. (1993) reported that there was a second MHC region, named the Rfp-Y system, which supported a strong similarity to the MHC, mapped to the opposite side of the nucleolar organiser region on the same microchromosome as the MHC. This region included two closely linked class I and two class II genes (Miller *et al*., 1994a). Recent work of Miller *et al*. (1996), using trisomy mapping had shown that the Rfp-Y system contained two class I and three class II genes and lied on chromosome 16, in the same cosmid cluster as a class II MHC gene and the rDNA genes (NOR). Between the two class I α genes placed a lectin, type C gene, *Lec* (Wain, 1997). The Rfp-Y system was a new polymorphic genetic region with high homology to class I and class II MHC genes. The Rfp-Y genes were highly similar to each other (93%) and to classical class Iα genes (73% with chicken class I and 49% with HLA-A). The 17.5 gene, together with its linked MHC genes, may be part of the recently described Rfp-Y (restriction fragment pattern Y) system (Bernot *et al*., 1994).

Juul *et al.* (1997) reported that one way mixed lymphocyte cultures using the polymorphic RFLP genotypes showed functional differences among the Rfp-Y haplotypes. Nucleotide sequence information of the B^1 domain of the Rfp-Y haplotypes supported the functional data. MHC like genes and haplotypes that segregate independently of the B complex, named Rfp-Y, had recently been reported

in fowls. To determine whether Rfp-Y genes controlled transplantation responses, trials were managed to determine if reciprocal skin graft rejection time and/or the magnitude of response in the 1 way mixed lymphocyte reaction (MLR) differed between fowls having identical vs. different Rfp-Y genes. Rfp-Y histocompatibility did not cause significant MLR responses. Nevertheless, Rfp-Y incompatible skin grafts were rejected more frequently and at a faster rate than Rfp-Y compatible grafts by 2 week old birds. Control MHC B incompatible grafts were rejected faster than Rfp-Y incompatible grafts. The results indicated that Rfp-Y class I and II MHC like genes are associated with the expression of minor histocompatibility antigens in fowls (Pharr *et al*., 1996).

MHC Identification

Briles *et al.* (1950) determined allelic differences of the chicken B complex by serological differences in blood cells. Blood group antigens in chicken had been studied originally by using alloantisera in a hemagglutination assay. The alloantisera were produced by immunization with erythrocytes between chicken differing at one or more blood group loci. The final antisera might contain antibodies against several antigens. It might be useful to make the antisera more specific by adsorptions with erytrocytes to remove particular antibodies from the solution. System A and system B, were the first to be identified genetically and serologically (Briles *et al.*, 1950). Two additional blood group systems, C and D, were characterized next and were shown to separate independently of each other and of the A and B system (Briles *et al.*, 1959 ; Briles, 1951 ; Briles, 1987 ; Briles *et al.*, 1963). Next, a fifth blood group system E was detected and shown to be closely linked to the A system (Briles *et al.*, 1959). Additional blood groups had been characterized, bringing the total number up to 13: A, B, C, D, E, H, I, J, K, L, N, P, and R. The significant varier of blood group allelic frequencies concurrent with selection for immune response or disease resistance suggested that some blood group systems might have important but as yet undiscovered functions (Johnson and Edgar, 1984; Dunnington *et al.*, 1984; Martin *et al.*, 1986). The R system had an allele associated with susceptibility to subgroup B leucosis sarcoma viruses (Crittenden *et al.*, 1970). The B blood group, although, promoted as a marker for the major histocompatability complex (Briles, 1987). The L system seemed to be acting in a manner independent of the B complex in response to RSV challenge (LePage *et al.*, 2000).

After Briles (1987) found serotyping method to identify MHC haplotypes, this method was used by haemagglutination of the BG antigens (and in some cases BF antigens), but give no information for the other MHC loci. These antigens were developed by monoclonal antibody method from known MHC haplotypes. Even with the high degree of polymorphism occurring within and between regions, general typing of erythrocytes for MHC haplotypes could still be performed economically with alloimmune reagents of known specificity.

Crone *et al*. (1981) found that two specific alloantisera detecting BL antigens on fowl lymphocytes of the B^6 and B^{15} haplotypes were found to cross react strongly. It appeared consequently that the BG locus was similar in its degree of polymorphism

to the locus whose alleles controlled class I cell membrane antigens. The standard haplotypes were almost all of White Leghorn derivation. Preliminary typings of other breeds of domestic fowls, and of wild Gallus species, indicated the existence of a much wider spectrum of allomorphs than that recognised to date (Simonsen *et al*., 1982). Near to total identity could be assured by cellular in vivo GVHR assays (Pazderka *et al*., 1975) or by in vitro mixed lymphocyte responses (Bacon, 1987). The biochemical methods enabled accurate definition of expressed MHC products, and could be a useful tool for the identification of B alleles in other lines or outbred fowls (Hepkema *et al*., 1991). There was a high correlation between these RFLP types and the serological B typing, since the RFLP type was identical within each pair of homozygotes. The RFLP patterns of F_2 birds accorded with the serologically determined B haplotypes of the birds, demonstrating the Mendelian inheritance of the polymorphic bands (Warner *et al*., 1989). Given good serology to identify BF antigens, it would still be desirable to confirm that the BF region is identical in two strains by another method. An additional highly sensitive and accurate method currently under development would use restriction fragment length polymorphisms (RFLP) of genomic deoxyribonucleic acid (DNA) hybridized with complementary DNA probes complementary for BL genes (Bacon, 1987), or BF genes. The developing B congenic strains were exceptionally good at producing high titred anti BF sera through immunization of different heterozygous recipients with lymphoid cells (Ewert *et al*., 1980; Simonsen *et al*., 1982). The well-known method of MHC haplotype identification was molecular typing of BG genes, using restriction fragment length polymorphism (RFLP) assays (Miller *et al.*, 1988). However, RFLP method had been limited by radioactive used. To identify MHC haplotype by BG RFLP with non-radioactive was not sensitive (Miller, *pers.comm.*). To solve this problem, single stranded conformation polymorphism (SSCP) of BF and BL genes were developed to use for MHC haplotype identification (Goto *et al.,* 2002). This method was very suitable and easy to use in many laboratory without radioactive. In addition, microsatellite markers LEI0258 was used to identify MHC haplotype. It placed on microchromosome close to BLβ gene.

MHC hoplotypes

In chicken, there were over 27 different MHC haplotypes reported and some of them controlled the response to economically important diseases including fowl cholera, salmonellosis, coccidiosis and Marek's disease (Briles and Briles, 1982; Briles *et al*., 1982). In Leghorns, 27 standard haplotypes had been verified by serological means. Involvements of disease resistance/susceptibility with the chicken MHC were investigated using White Leghorns and experimental chicken lines because their MHC haplotypes were well defined (van der Zijpp, 1983; Lamont,1989). Since alloantisera used to verify B serotypes in Leghorn lines did not work well outside the line in which they were raised, a panel of B alloantisera was produced to identify B serotypes within a commercial broiler breeder line of fowls (Li *et. al*., 1999). Genomic DNA was analyzed to verify the MHC molecular genotypes in three parental broiler lines used in a three way cross to produce a emulative broiler chick. Afterward, identification of MHC genotypes, broiler chicken with defined genotypes was immunized to produce antisera. The DNA sequences of broiler chicken

Class II MHC genes was attained and compared to previously reported Class II genes seperated from standard chicken MHC haplotypes. Other investigators had used BG RFLP comparisons to define broiler B haplotypes (Landesman *et al*., 1993), and some success had been performed in typing true haplotypes in meat type chicken by using alloantisera specific for Leghorn B haplotypes (Heller *et al*., 1991) or B subregion specific sera (Simonsen *et al*., 1982). The serologically defined B^{21} haplotypes could be divided into 5 subtypes defined by 7 BL and BF RFLPs; only 1 of the fragments was B^{21} specific (Chausse *et al*., 1989). There were analogies and differences in disease resistance and immune response among B haplotypes. These haplotypes could all be divided by molecular typing of BG genes, using restriction fragment length polymorphism (RFLP) assays (Miller *et al.*, 1988). The RFLP typing with the clone of BG cDNA was qualified to analyze the B haplotype in more detail than typing by haemagglutination (Lamont *et al*., 1990; Nishibori *et al*., 2000). The B homozygotes were identified serologically and compared by BG genotyping using RFLP analysis. Reverse transcription PCR was used to amplify variable domains of expressed BLβ and BF genes of homozygotes of most of the B serotypes (Li *et al*., 1999). The BG antigens were highly polymorphic, and were encoded by genes located within the MHC of fowls, the B system, and were found only on erythrocytes; they conformed to neither class I nor class II antigens (Goto *et al*., 1988). However, ascertainment of the B genotype by this method was difficult in the randomly bred population. The compound polypeptide pattern confirmed the serological proof for a partial duplication within the BG subregion, and contributed indirect evidence for (1) the occurrence of multiple loci within BG, and (2) a means by which polymorphism might be introduced into the MHC of fowls (Miller *et al*., 1988).

Similar MHC haplotypes had virtually identical patterns and levels of expression at the protein level. That first observation showed that the haplotype B^{21} was resistance and B^{19} was susceptibility markers to MD (Hansen *et al*., 1967). Afterward studies primarily accepted with the finding that B^{21} was the resistance haplotype and also showed that the B^2 haplotype had the same effect. Viremia levels at day 5 and 6 postinfection were certained to be lower in chicken supporting the B^2 haplotype compared to those with the susceptibility haplotypes (Bacon, 1987).

The genetic differences within the B complex had been involed with resistance to a number of diseases; the initial association of the MHC was with MDV resistance (Table 1). The B^{21} haplotype had been shown by a number of experimenters to confer resistance to MDV (Hansen *et al*., 1967; Briles *et al*., 1977; Powell, 1984). However, the B^{19} and B^2 haplotypes conduced to confer susceptibility. Bacon (1987) found some resistance associated with the MHC; as haplotypes B^2 or B^{21} were more resistant than haplotypes B^5, B^{15}, or B^{12}. However, Bumstead *et al*. (1993) examined 11 inbred and partially inbred lines of chicken and determined by F_1 and F_2 crosses that resistance was partially dominant and did not involve the MHC. Furthermore, MHC haplotypes involed with production traits (Table 2).

Table 1 Diseases in which an association with B antigens was investigated

Conditions[1/]	*B* haplotypes[2/]
Marek's disease	
Macerated tissue at 1 day or field exposure:Hy-Line hybrids	19 ; 21
Field exposure:DeKalb hybrids	2 ; 6 ; 13 ; 19
JM-MDV[3] at 2 weeks:JM-N x JM-P crosses	13 ; 19 ; 21
LM-MDV at 2 weeks:Regional Cornell Random bred	13 ; 15 ; 5 ; 19 ; 21
Exposure of chicks to BC-1-MDV Shedder Chick :	
ED-1 (F_2 hybrid)	2 ; 21
ED-2 (F_2 hybrid)	21 ; 14
BC-1-MDV homogenate at 3 weeks:	
Ottawa x R line	2 ; 21
Ottawa x P line	2 ; 21
Ottawa x N line	2 : 21, X*
HPRS-16-MDV at 3 weeks:commercial cross	2/21 ; 5/21 ; X/21 ; 15/21 ; 19/21
JM-MDV at 2 weeks:6_3 x 15_1 F_5 cross	2 ; 5
JM-MDV at 2 weeks:DeKalb hybrids and recombinant cross	21/19 ; 19/19 ; F21-G19/19
JM-10-, GA-5-, or RB-1B-MDV at 2 days: UCD-003 inbred and 7 B congenic lines	2 ; 15 ; 17 ; 18 ; 19 ; 21 ; Q* ; 24
Exposure to MDV-Shedders chicks at 1 to 14 days	Failure to correlate resistance with B type in several lines of varying resistance
Transient paralysis	
GA-MDV at 3 to 4 weeks and paralysis after 8 days:G-B1 x G-B2 hybrids	13 ; 6
Sarcoma tumors	
BH RSV[4] (RAV-1) at 6 weeks in wing-web -6_1 x 15_1 F_2 hybrids	2 ; 5
Schmidt-Rupin RSV at 4 weeks in wing-web:G-B1 x G-B2 hybrids	13 ; 6

Table 1 (continued)

Conditions[1/]	*B* haplotypes[2/]
Erythroblastosis	
Avian leucosis virus (RAV-1) at 1 week : 6_3 x 15_1 F_5 hybrids	2 ; 5
Lymphoid leucosis	
Avian leucosis virus (RAV-1) at 1 week : 6_3 x 15_1 F_5 hybrids	2 ; 5
Spontaneous autoimmune thyroiditis	
Thyroid pathology and antibody to thyroglobulin : obese strain (OS) inbreds (Cornell)	13 ; 15 ; 5
OS x CS F_2 (Cornell)	6 ; 15
Coccidiosis	
$15I_5$ B congenic lines *Eimeria tenella* or E.acervulina	15 ; 5 ; 2 ; 12 ; 13 ; 19
Auburn line A selected for resistance (R) And susceptibility (S) to *E. tenella* (1948 to 1959)	Three alleles in moderate (f) in S line absent in R line. R line had 2 high (f) alleles, one absent in S, the other in lower(f) in S
6_1 x 15_1 F_4 Crosses challenge of immunized chicken with *E. tenella* cecal lesions and delayed wattle reaction	5/5 ; 2/5 ; 2/2

[1/] White Leghorn strains unless otherwise indicated.
[2/] Standard nomenclature of Briles *et al.* (1982) except where astrisk indicates a tentative symbol. The types with underline or no underline indicate relatively resistant (regressor of sarcoma) and susceptible genotypes, respectively. (f) = gene frequency.

1. MDV = Marek's disease virus.
2. RSV = Rous sarcoma virus.

Source: Bacon (1987)

Table 2 Productivity traits where an association with B antigens was observed

Conditions	B haplotypes[1/]
Fertilization rate-Light Sussex strain 6D.	
Light Sussex strain 6D.	38/38* ; 35/38 ;
Two other strains with no, or confounded differences	36/38 ; *35/36* ; 37/38 ; 36/36
Embryonic mortality	
Light Sussex	35/37* ; 35/38 ;
Thornber strain 6	36/37 ; *35/35* ; *37/38* ; *38/38* ; 36/38 ; 36/36 ; 35/36 ; 37/37
Hatchability	
Three inbred lines	homozygous vs. heterozygous mating
B-Congenic lines	19/19 best of 8 B congenic lines
Juvenile livability and egg productivity:	
H10	13/13 ; 13/14 ; 14/14
Adult mortality and egg productivity:	
H3	6/6 ; 6/7 ; 7/7 2/2 ; 2/14 ; 14/14
Long life span in F_2 crosses	
Adult mortality and egg productivity	
H1	2/2 ; 1/2 ; 1/1
Iowa S1	2/2 ; 21/21 ; 19/19; heterozygous ; 1/1
B-congenic lines	2/2 lowest of 8 lines ; 2/Q* lowest of heterozygous
Egg production	
Ottawa Strain 7 selected for high egg Production in substrains 8 and 9	(f) of 21 increased ; 19, 2 and 12 retained ; 15, 13 and Y decreased
Ottawa Strain 8R selected for MD resistance and egg productivity	(f) of 2 and 21 increased ; 19 and 12 decreased ; 15, 13, Y eliminated

Table 2 (continued)

Conditions	B haplotypes[1/]
Selection for egg production using low protein diet	15 favored over 19
Iowa State line selected for high egg mass and body weight vs. control	(f) of 2 and 14 increased; 13 decreased
Iowa State line selected for high egg mass : feed consumed vs. control	(f) of 2 increased ; 13 decreased ; 14 retained

[1/] Standard nomenclature of Briles *et al.* (1982) except where * indicates a tentative identification symbol. The types with underline, reclined, or no underline indicate favorable, intermediate, and relatively unfavorable genotypes, respectively. (f) = gene frequency.
Source: Bacon (1987)

Emara *et al.* (2002) studied in broilers. The results showed that genetic selection by the primary breeder contributed sufficient genetic diversity, because broilers were continueing progress in production traits. In this manner, the result from Ewald and Livant (2004) reported less polymorphism in MHC class I, because the conservation of the alpha helix of the alpha 1 domain related to MHC interaction with members of the killer immunoglobulin-like receptors on NK cells that were specific for recognition of MHC molecules and function to regulate activation of NK cells. Whereas MHC molecules might be dominant ligands for NK cell regulation, MHC molecules were more important in presenting antigen to cytotoxic T lymphocytes. By this way, broiler population had sufficient genetic diversity in disease resistant traits.

Mechanism of MHC

Abbas *et al.* (2000) reported that all cells in the body exhibited MHC class I molecules and when cells were infected with a virus or other intracellular pathogen, they displayed fragments of the antigen on the MHC class I molecules (Figure 7). T cells recognized the MHC fragment complex and signal the cell displaying the complex to die. This was a good reaction because most cells did not have any way of producing antibodies to fight infection. Clearly if they could produce antibodies, the antibodies could not function correctly because the pathogen would be inside cells. Killing the infected cell allowed the body to destroy a sick cell quickly to save the healthy cells around it; leaving the infected cell exist while trying to find an efficient way to destroy the infection would probably do more damage than good. A distressed cell had to confide mainly on the actions of so called B cells and helper cells or killer T cells. While the B cells with a bit of help from T cells could MHCs orchestrate a guided missile attack on aggressors get to outside our cells, such as bacteria, they could not work readily expel viruses, which ambush inside the cells. It dropped to the so called killer T cells to seek out virus infected cells and eradicate them from our system. However, like normal proteins inside the cell, viral proteins were taken apart from time to time, and their pieces picked up by MHC class I proteins. When an MHC class I protein presented viral fragment to a killer T cell, the killer kicked into action

and destroyed the pirated cell and hopefully the virus with it. Cytotoxic T cells later became active cytoxic T cells as they matured. Proteins secreted or released in infected cells were a major source of peptides for MHC class I presentation and for the generation of parasite specific CTL (Abbas *et al*., 2000).

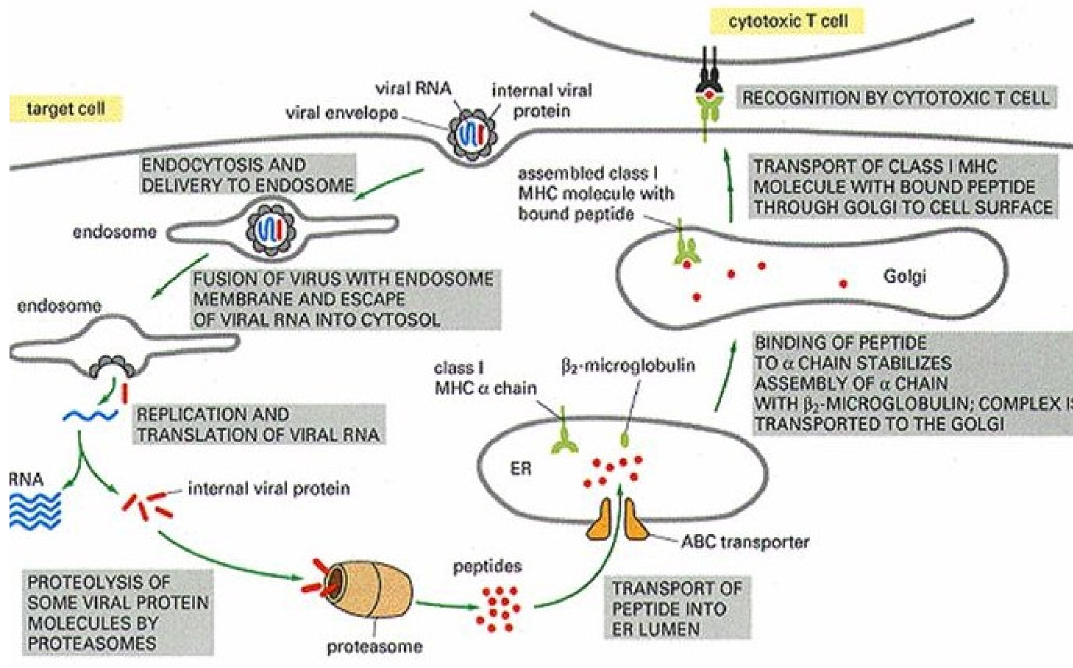


Figure 7 Pathways of antigen processing and presentation. In the classI MHC, protein antigens in the cytosol are processed by proteasomes, and peptides are transported into the ER, where they bind to classI MHC molecules.
Source: Abbas *et al.* (2000)

Abbas *et al*. (2000) reported that the molecules of MHC class II were cell surface glycoproteins which bound antigenic peptides and presented them to T lymphocytes for immune recognition (Figure 8). MHC class II molecules were heterodimers with an antigen binding site containing the outermost domains of both subunits, each of which cooperated four strands and one helix to form the base and sides of a cleft capable of binding a peptide of up to 15 amino acids in length. An important lineament of the antigen binding site (ABS) of MHC molecules was its very high level of polymorphism, with alleles differing by 10-20 amino acids within the 200 residue domain. Different MHC alleles consequently presented different populations of peptides to T cells, which might or might not include peptides important for the evolution of an efficient immune response. Placing on the surfaces of cells, MHC molecules bound and exhibited a protein fragment for T cell recognition. Both classes of MHC hold protein fragments but the sources of the protein fragment varied. The molecules of MHC class I derived the protein fragment they displayed from intracellular proteins. In contrast, MHC class II molecules displayed fragments of extracellular protein. T cells could either kill the cell displaying the complex (class I activation) or activate it (class II activation) to start

rising a defense against the pathogen. In this way, B cells digested some of the antigen and exhibited its protein fragments on the surface on MHC class II molecules. If a T cell recognized the MHC fragment complex, it informed the B cell to create antibodies and to abound (Abbas *et al*., 2000).

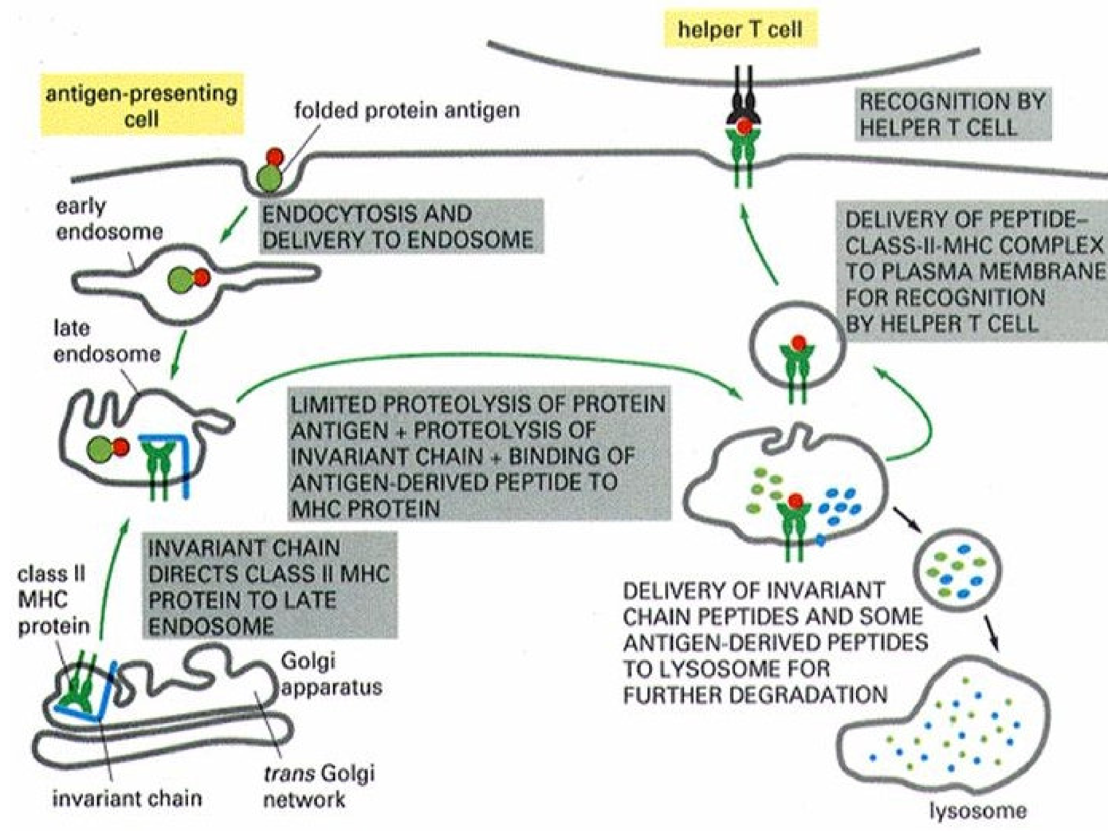


Figure 8 Pathways of antigen processing and presentation. In the classII MHC, extracellular protein antigens are endocytosed into vesicles, where the antigens are processed and the peptides bind to classII MHC molecules.
Source: Abbas *et al.* (2000)

Proteins of MHC class I and II instanced these fragments and pick up the pieces like tweezers: MHC class I harvesting the fragments from proteins that were inside the cell and MHC class II collecting those that came from outside. The MHC proteins then passed to the cells membrane and exhibited their cargo at its surface for inspection by T cells, a procedure called antigen presentation. Helper and killer T cells circulated through the body 24 hours per day, scrutinizing the contents of MHC proteins, and then getting updates on what was happening when and where. When helper T cells pointed an MHC class II protein carrying this classifier of bacterial debris, they were turned on, and encouraged B cells to make antibodies. These antibodies tagged the bacteria ensuring that they were eliminated from the body (Abbas *et al*., 2000).

Depending on the molecules that appeared on the surface of the cell (CD4+, CD8+), the MHC class II cells that the immune cell could interact with became

known. Remember that the MHC class II cells cooperated in presenting antigens to the T cells, which allowed them to lead a counterattack against pathogens. Therefore, the molecule presented on the surface of the cell directed to regulation of which T cells partook in an combat against a particular pathogen. MHC class II molecules bound to fragments of endocytosed protein during their transported to the cell surface. There the complex of MHC class II molecule and peptide antigen might be recognized by receptor molecules on T cells, which were specific for both the MHC molecule and the bound peptide (Abbas *et al*., 2000).

Avian Influenza Virus Disease

Genome analysis of Avian Influenza Virus

Avian influenza was defined as fowl plague in 1878 as a disease causing high mortality in chickens in Italy. It was not until 1955 that it was demonstrated that fowl plague was an avian influenza virus whose genomic composition was effectively identical to the one found in the human influenza virus (Bronze and Greenfield, 2005). Avian influenza virus disease was caused by avian influenza virus (AIV) type A, capsulated RNA virus of Orthomyxoviridae family. It had 2 specific surface antigens, haemagglutinin (H, H1-H15) and neuraminidase (N, N1-N9). Influenza viruses consisted of 3 types, 1) Type A was composed of 15 subtypes of H and N antigens. They were found in swine, horse, human, and poultry. 2) Type B was found in human. 3) Type C was found in human and swine (Lee *et al,* 2001, Department of Livestock Development, 2004, Hanson, 2004). The viruses placed in the digestive and reproductive tracts of the birds and in shed from both these sources during the early stages when the disease was acute. When the virus was highly pathogenic, it became systemic and invaded practically all organs. Like most disease-producing organisms, the virus survived in most and cool conditions, but it tended to die out in dry conditions and under high temperature. The neuraminidase of type A AIV in human and turkey found in Dutch, Scotland, England, and USA in 1930-1970 were related antigenicity. This result indicated that the recurrence of a neuraminidase antigen in avian influenza A viruses over a period of at least 40 years. The haemagglutinins of them didn’t cross react particularly with each other (Kendal *et al*., 1971). Some of subtypes in type A was distantly and closely related between poultry and mammal. The H gene in poultry, isolated from Israel, was more distantly related to H1N1 swine viruses isolated in the USA or Italy. The neuraminidase of this isolate was closely related to other N2 neuraminidases from avian and human isolates. It indicated that in Israel the virus N gene was uncommon related among avian influenza A strains (Lipkind *et al*., 1984). Neuraminidase of avian influenza A virus reformed greatly from one strain to another in substrate particularity as compared with those of human influenza A and B viruses, and that some strains of avian influenza A virus (Figure 9) had a neuraminidase with unique enzymological individualities different from that of human influenza A virus as well as that of influenza B virus (Kiyotani *et al*., 1987).

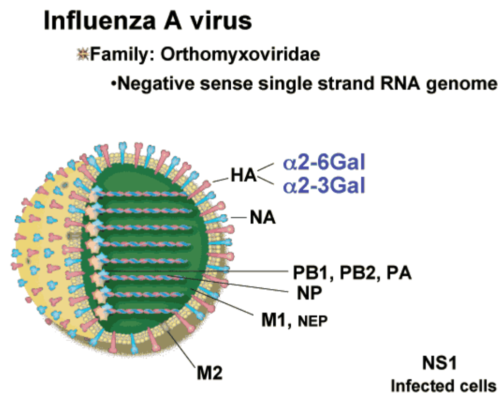


Figure 9 Influenza A virus
Source: Bronze and Greenfield (2005)

Influenza virus attachment to the susceptible cell was mediated by the interaction between the viral hemagglutinin and sialic acid receptors present on glycolipids and glycoproteins on the cell surface. At this stage, the sialidase activity of the neuraminidase prevents binding of the H to sialic acids present in mucopolysaccharadies, which would otherwise interfere with the virus binding to the adequate cellular receptors. The virus is internalized by endocytosis and, upon acidification of the endosome, conformational changes on the hemagglutinin lead to the fusion between the viral and the endosomal membranes. Acidification of the endosomal lumen also activates the ion channel activity of the viral membrane protein (Bronze and Greenfield, 2005). After this stage, MHC class I would play the role of immunological system (Abbas *et al,* 2000) (Figure 7 and Figure 10). After infection of AIV, viral replication led to cell death. Local symtomps were the result of cell death, and inflammatory response to infection, then there were increases of INF-β and –α, virus specific cytolytic T cell, and anti-influenza virus antibodies. These activities were associated with MHC class II (Abbas *et al,* 2000).

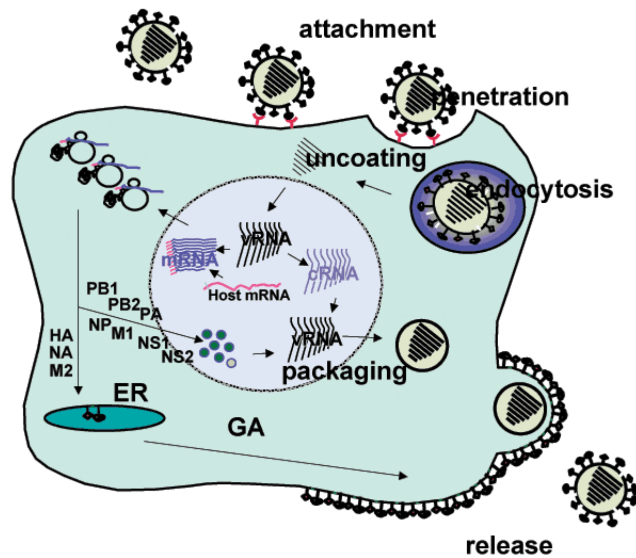


Figure 10 Life cycle of Avian influenza virus A
Source: Bronze and Greenfield (2005)

Manifestations of Avian Influenza Virus Disease

Hanson (2004) reported that the signs and symptoms varied, depending on the type of bird, the dose and the route of entry, but the disease could start anywhere from a few hours to up to two weeks after exposure, especially in flocks. Symptoms, which could imitate those of other diseases, included sneezing and coughing, increased broodiness, reduced egg production, reduced activity, reduced feed consumption, and sometimes an increased sudden mortality without any prior symptoms or causes. Other signs might include increased lacrimation, skin diseases, nervous disorders, and diarrhea. An infection might result in no illness, a mild disease, or a highly fatal disease. This might be due to the type of virus involved but also, of course, to the age, sex, and health of the birds themselves and whether they carried some other disease. An AIV could be highly pathogenic for one avian species but not for another. Like most diseases, some affected birds might show no clinical signs and simply become carriers and continue to shed the virus into the environment.

Situation of Avian Influenza Virus Disease Outbreaks in Thailand

During November 2003 to March 2004 in many country in Asia, such as Cambodia, China, Hong Kong, Indonesia, Japan, Korea, Loa, Vietnam, and Thailand, there were outbreaks of avian influenza virus, especially in Vietnam and Thailand, strain of H5N1, causing of death of infected patients and devastated poultry industry. The H5N1 strain had a different genetic sequence and therefore was believed to come from a different source. It was violent pathogenic Avian Influenza (FAO, 2004). In Thailand, there were ongoing outbreaks of avian influenza viruses (Department of Livestock Development, 2004). It devastated local economies and to both commercial poultry operations and smallholders. Chicken prices were down slightly last year in the EU. While a banned Thai chicken decreased chicken frozen meat in the market.

Thus, Brazilian, a competitive producer of similar type of product, moved to supply the gap. In the Japanese market, domestic poultry supplies dropped as avian influenza infected flocks were disposed of and imports were banned from Thailand (Thailand accounts for thirty percent of Japanese poultry imports). This pushed prices of all meats higher (FAO, 2004).

Avian influenza outbreaks were difficult to control and often caused major economic impacts for poultry farmers in affected countries, since mortality rates were high and infected fowl generally might be destroyed in order to prevent the spread of the disease. As a result of ongoing outbreak in Asia, FAO estimated that around 20-25 million birds had been destroyed (FAO, 2004). For this reason, the local economies of both commercial poultry operations and smallholders were devastated, especially in Thailand, where the industry was heavily reliant on trade. In 2003, poultry exports from Thailand accounted for nearly 7 percent of global poultry meat trade, with an export value of approximately US$1 billion (FAO, 2004). The effects of avian influenza virus disease outbreak severely decreased frozen poultry meat exportation. The most importance of this outbreak, it caused of death of infected patients in Thailand and Vietnam (FAO, 2004).

Problems of Outbreaks of Avian Influenza Virus Disease

After outbreaks of AIV, many of poultry were destroyed, because this disease could spread to human. Thus, there were huge losses of economics, i.e. in Thailand, frozen chicken meat exports was banned by market share in the world. In USA, Virginia agriculture officials had ordered more than 1.4 million chickens and turkeys destroyed as entire flocks contract avian influenza viral outbreak not seen in the state since 1983. The influenza, harmless to humans, was much like the human version of the influenza (Anonymous, 2002).

Prevention and Controlling of Avian Influenza Virus Disease

As the report of Hanson (2004), AIV could be held by rapid depopulation of affected flocks and quarantined of such farms, and the use of antibiotics to ward off secondary infections. Vaccinations might be used after the virus has been isolated and confirmed because there was no way of predicting which subtype will be responsible for an exposure. The problem with vaccines was that some vaccinated birds became infected and then became a source of the virus. Therefore, such flocks could help to spread the virus. As a result, use of vaccinations after the disease had actually occurred is not usual. Rapid, stringent methods and a dedicated application of biosecurity measures could help prevent the widespread dissemination of AIV once it got started. Complete destruction of any birds or flocks with clinical, serologic, or virologic evidence of the virus were recommend in order to ensure that any bird or flock with any kind of exposure was eradicated, eliminating a potential source of infection. The fact that a wide variety of the virus is carried by wild birds and waterfowl made it difficult to eradicate completely because there was always the chance that infected droppings from such sources could be introduced into backyard and commercial flocks. This was a very good reason why biosecurity measures on all

poultry commercial farms be strictly adhered to at all times. Under normal circumstances, it was always a good idea to separate avian species. Simply keeping commercial birds away from domestic and wild birds was a sensible idea. When AIV wass discovered in an area, one of the best lines of defense discouraged people who raised commercial poultry from congregating. This ensured that the virus was limited in how it can spread. The fact that a low-pathogenic avian influenza virus was responsible for an outbreak was no cause for complacency because the virus could circulate for months and then became highly pathogenic. The emphasis, therefore, was on immediate and total containment and eradication. However, low-pathogenic AIV could not become high-pathogenic AIV in defense lines of birds, so this was a good reason for AIV resistant genetic improvement in order to preventing suitiation, but it did't suit for controlling period.

The other way to prevent AIV studied by Swayne *et al.* (2003), reported that avian influenza viruses were major contributors to viral disease in poultry as well as humans. Outbreaks of high pathogenicity avian influenza viruses caused high mortality in poultry, resulting in significant economic losses. The potential of avian influenza viruses to reassort with human strains resulted in global pandemics in 1957 and 1968, while the introduction of an entirely avian virus into humans claimed several lives in Hong Kong in 1997. Despite considerable research, the mechanisms that determine the pathogenic potential of a virus or its ability to cross the species barrier were poorly understood. Reverse genetics methods, i.e., methods that allowed the generation of an influenza virus entirely from cloned cDNAs, had provided with one means to address these issues. In addition, reverse genetics was an excellent tool for vaccine production and development. This technology should increase the preparedness for future influenza virus outbreaks.

<u>Resistant Traits to Avian Influenza Virus Disease</u>

Lyon and Hinshaw (1991) reported that the virulent avian influenza virus A/Ty/Ont/7732/66 (H5N9) (Ty/Ont) causes a rapid destruction of lymphoid cells in infected birds. Avian macrophage cell lines, HD11 and MQ-NCSU, support productive replication of Ty/Ont and other influenza viruses. Therefore, the ability of these cell lines to produce nitric oxide (NO), a potentially cytotoxic mediator, in response to infection with Ty/Ont was examined. Although treatment with bacterial lipopolysaccharides (LPS) resulted in high NO levels, infection of macrophages with Ty/Ont resulted in NO levels lower than in untreated cells. Ty/Ont was also able to inhibit the positive response to LPS in cultures simultaneously treated with LPS and virus. However, inactivated influenza virus did not exhibit this inhibitory effect. Different strains of influenza virus varied in their ability to inhibit NO production by the macrophages; this might be related to the level of virus replication in these cells. It was concluded that the ability of the avian macrophage to activate the NO synthesis pathway was seriously diminished by infection with virulent influenza viruses such as Ty/Ont. NO level was related with some genes expression associated with disease resistant traits in animal (Abbas *et al.,* 2000).

Genetics of Disease Resistance

MHC Genes or linked markers could be used as genetic markers for disease resistance selection, because this region had been confirmed to influence genetic resistance to MD. Some MHC class II bands were associated with production traits or with MD resistance, and that these associations tend to be unique to each genetic background. Hence, MHC class II genes were likely candidates for the investigation of quantitative trait loci in egg production and disease resistance traits (Lakshmanan *et al*., 1997). However, it was clear that other non MHC genes were involved and fact comprise the majority of disease resistance. Although genetic resistance was complex and controlled by several quantitative trait loci (QTL), selection for high levels of resistance could be obtained within relatively few generations (Cole, 1968). This approached to enhance genetic resistance to AIV through MAS assumes that the appropriate markers for AIV resistance genes had been precisely located on the chicken genome.

The genetic control of AIV response was also of increasing interest as ameans to improve the efficiency of vaccination against this disease similarly to other diseases. Bacon and Witter (1994) found that the degree of protection after vaccination against MD was influenced by the MHC, and there was an interaction between the MHC (*B*) haplotype and the vaccine serotype. There is no data of genetic resistance to AIV now. After AIV outbreak in Thailand, some Thai indigenous chickens were survived. They were suggested that they were resistant to AIV.

MATERIALS AND METHODS

1. Chicken

Two groups of chicken were used in this study:

1.1. Two hundred and fifty five of Thai indiginious chicken kept at Livestock Research and Breeding Center of Department of Livestock Development (DLD), composed of LHK, PHD, CH, ND, and KL chicken. These Thai indigenous chickens were developed by DLD. They were bought from every part of Thailand. They were raised and developed at Livestock Research and Breeding Center of DLD more than 20 years. In this manner, they were the reference population of Thai indigenous chicken.

1.2. Seven hundred and thirty Thai indigenous chicken in rural area of central part of Thailand in the area of AIV outbreak. They were kept from small holder farms that there were dead and survivable chicken in each farm. Chicken blood from each farm was collected to test titre of AIV.

2. Materials for laboratory analysis

2.1. Chemicals and materials

Blood collecting	Hypodermic needle, microcentrifuge tube, 75% alchohol, 0.5M EDTA, Freezer box, glove, label marker
Feathers collecting	Plastic bag, label marker
DNA extraction	Proteanase K, NaCl, Tris-HCl (pH 8.0), EDTA, SDS, saterated phenol, chloroform, isoamyl alcohol, 95% ethanol, 70% ethanol, distrilled water
DNA concentration Measurement	Lamda DNA, agarose gel, bromophenol blue, xylene cyanol FF, TBE buffer
PCR method	Taq polymerase, $MgCl_2$, dNTPs, 96 well PCR plate, automatic pipette, cooler box, PCR box, PCR lid
Electrophoresis	Agarose gel, denatured polyacrylamide gel, bromophenol blue, xylene cyanol FF, TBE buffer, silver staining, APS, TEMED
SSCP	Non-denatured polyacrylamide gel, bromophenol blue, xylene cyanol FF, TBE buffer, silver staining, TEMED

2.2. Reagents

All solutions used in this investigation were prepared with deionized and demineralized water.

Solution	Component	Amount
Acrylamide (acrylamide:bis-acrylamide, 19:1) (40%)	Acrylamide	95.00 g
	Bis-acrylamide	5.00 g
	Water added to	250.00 ml
Denatured acrylamide gel (4.5%)	Acrylamide 40%	11.25 ml
	5x TBE	10.00 ml
	Urea	42.00 g
	Water added to	100.00 ml
Non-denatured acrylamide gel (12%)	Acrylamide 40%	30.00 ml
	5x TBE	20.00 ml
	Water added to	100.00 ml
Silver staining solution	Silover nitrate	1.00 g
	Water added to	1000.00 ml
	Formaldehyde	1500.00 µl
Developing solution	Sodium carbonate	30.00 g
	Water added to	1000.00 ml
	Formaldehyde	750.00 µl
	$Na_2S_2O_3$ 2 mg/ml	500.00 µl
TBE(5x) buffer	Tris	54.00 g
	Boric acid	27.50 g
	EDTA (0.5 M)	20.00 ml
	Water added to	1000.00 ml
TE buffer	Tris (1 M)	10.00 ml
	EDTA (0.5 M)	2.00 ml
	Water added to	1000.00 ml

2.3. Used softwares

BLAST program	http://www.ncbi.nlm.nih.gov/blast/
SAS version 6.12	SAS Institute Inc., Cary, NC
POPGENE version 1.31	Francis *et al* (1999)

2.4. Equipment

Centrifuge	Spectrafuge national labnet co.

Electrophoresis (agarose gel)	Gelmate 2000, toyobo
Electrophoresis (polyacrylamide gel, vertical)	Hoefer miniVe
Gel dryer	BioRad
PCR thermocycler (PTC100)	MJ Research, USA
Power supply	Electrophoresis power supply eps 301, Amersham
UV-Transilluminator (tcx-20)	Vilber lourmat
Autocrave	Sanyo
Hot air oven	Dekthai

3. Chicken DNA extraction

Blood and feather samples were collected from two groups of chicken, Thai indigenous from DLD as a reference population and Thai indigenous chicken from outbreak area. They were used to extract genomic DNA. Extraction of DNA from blood was performed by 0.5 ml of chicken blood collected from the wing vein and kept in micro tube with EDTA. Blood was treated by lysis buffer, consisted of NaCl, Tris-HCl (pH 8.0), EDTA, SDS, left in 55°C incubator 10 minutes, and added by proteinaseK, left in 55°C incubator 2 hours. The samples were treated with the combination of phenol: chloroform: isoamyl alcohol (25: 24: 1), mixed, and separated. The upper layer were phenol-extracted, centrifuged, upper layer collected, and added by 95% ethanol, DNA strand were collected in micro tube, washed by 70% ethanol two times, and left to removed ethanol (Sambrook *et al.* 1989). The DNA samples were added by TE buffer, consisted of 10 mM Tris and 1 mM EDTA, pH 7.0, and kept at –40°C.

DNA extracted from feather was performed by using 50 mg clean individual tip feather. Tip feather was cut into small pieces and soaked in guanidine solution, left in 55°C incubator >18 hours, and added by proteinaseK, left in 55°C incubator 2 hours. The samples were treated with the combination of phenol: chloroform: isoamyl alcohol (25: 24: 1), mixed, and separated. The upper layer were phenol-extracted, centrifuged, upper layer collected, precipitate by 2 times volume of 95% ethanol added with 10% of 3M sodium acetate, and 12000 rpm centrifugation. Precipitant was collected and washed by 70% ethanol two times, and left to removed ethanol (modified from Sambrook *et al.*, 1989). The DNA samples were added by distrilled water, and kept at –40°C.

Each DNA sample was concentration measured by the comparision between the concentration of DNA band of agarose gel and standard concentration. Genomic

DNA sample were mixed with steamed distilled water for appropriate concentration (20 ng/μl), and kept. These DNA were used in PCR methods of primers in Table 3.

Table 3 Primer pairs for microsatellite markers, BF, and BLβ genotyping

Region	Primer Length	Sequence[1]	Anneal Temp[2]	Fragment Size
LEI0258-F	22	CACGCAGCAGAACTTGGTAAGG	59°C	205 bp
LEI0258-R	22	AGCTGTGCTCAGTCCTCAGTGC		
CAJF01-F	23	TCGGGAAAAGATCTGAGTCATTG	64°C	311 bp
CAJF01-R	21	GATTTTCAGATCGCGTTCCTC		
BFα1-F	18	GTGGACGGGGAACTCTTC	60°C	178 bp
BFα1-R	21	TCTGGTTGTAGCGCCGCTGCA		
BFα4-F	18	GTGGACGGGGAACTCTTC	60°C	186 bp
BFα4-R	18	ACCGCCGGTCTGGTTGTA		
BLβ-F	18	GTGCCCGCAGCGTTCTTC	60°C	277 bp
BLβ-R	18	TCCTCTGCACCGTGAAGG		

[1] Primer sequence from McConnell *et al.* (1999), Fulton *et al.* (2004), and Goto *et al.* (2002)
[2] This temperature is approximately minus by 5°C

4. MHC Class II Haplotypes Identification by Using LEI0258 Microsatellite Marker

Genomic DNA from chickens, extracted by previous method were used to screen MHC haplotype by PCR of microsatellite marker, LEI0258. DNA samples were amplified by using primers of LEI0258 (Table 3) (McConnell *et al.,* 1999). This microsatellite was tetranucleotide microsatellite near BLβ I on microchromosome 16 (Figure 11). Each 15 μl reaction contained 20 ng of DNA, 0.5 μM of each primer, 1.9 mM $MgCl_2$, 0.2 mM of each dNTP and 1 unit of *Taq* DNA polymerase in the supplied buffer (final concentration : 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0, 0.01%(w/v) Tween). PCR amplification of DNA was performed using Touchdown PCR in a PTC-100™ Programmable Thermal Controller using the reaction profile of initial denaturation of 94°C 3 min, denaturation of 94 °C 0.45 min, annealing 59 °C 0.45 min, temperature increment -1 °C/cycle, extention of 72 °C 1 min, for 5 cycle, denaturation of 94 °C 0.45 min, annealing 54 °C 0.45 min, extention of 72 °C 1 min, for 25 cycle, and final extention of 72 °C 10 min.

PCR products were analyzed polymorphism by electrophoresis of 4.5% polyacrylamide gel and silver nitrate staining. The gels were imaged composed with Adobe Photoshop.

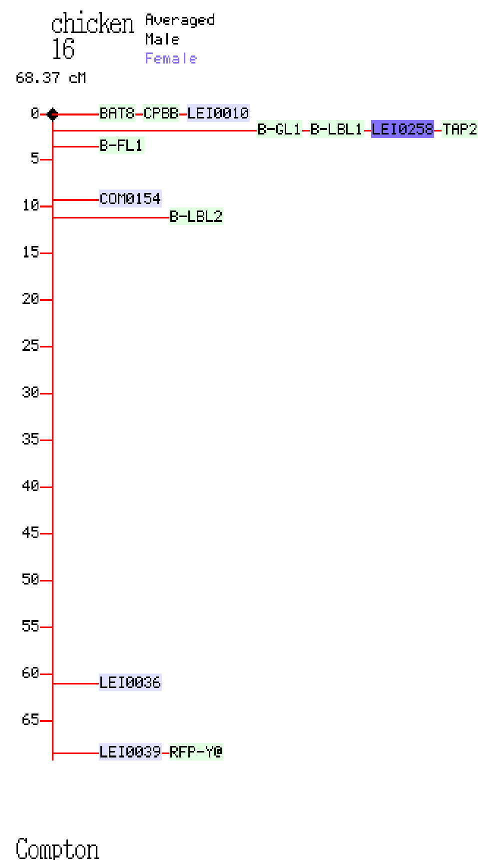


Figure 11 Microsatellite markers and genes on microchromosome 16 of female chicken of Compton population

Source : Roslin Bioinformatics Group (1999)

Each group of PCR products from LEI0258 was standardized by partial sequencing of BLβII genes to identify MHC haplotype. DNA samples were amplified by using forward and reverse primers of exon2 of BLβII genes (Table 3). Each 20 µl reaction contained 20 ng of DNA, 0.5 µM of each primer, 1.9 mM $MgCl_2$, 0.2 mM of each dNTP and 1 unit of *Taq* DNA polymerase in the supplied buffer (final concentration : 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0, 0.01%(w/v) Tween). PCR amplification of DNA was performed in a PTC-100^{TM} Programmable Thermal Controller using the reaction profile of initial denaturation of 94°C 3 min, denaturation of 94 ^{o}C 0.45 min, annealing 54 ^{o}C 0.45 min, extention of 72 ^{o}C 1 min, for 35 cycle, and final extention of 72 ^{o}C 10 min. Aliquots of the PCR reactions were checked for robustness using 2% agarose gel electrophoresis before automated sequencing.

Each group of PCR products from LEI0258 was sequenced by using primers of CAJF01 microsatellite marker to detect how this microsatellite identified MHC haplotype. The CAJF01 marker lied outside LEI0258 marker. Genomic DNA from chickens was amplified by PCR of microsatellite marker, CAJF01 (Table 3) (Fulton *et al,* 2004). This microsatellite lied on microchromosome 16 outside LEI0258. Each 15 µl reaction contained 20 ng of DNA, 0.5 µM of each primer, 1.9 mM $MgCl_2$, 0.2 mM

of each dNTP and 1 unit of *Taq* DNA polymerase in the supplied buffer (final concentration : 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0, 0.01%(w/v) Tween). PCR amplification of DNA was performed in a PTC-100^{TM} Programmable Thermal Controller using the reaction profile of initial denaturation of 94°C 3 min, denaturation of 94 ^{o}C 0.45 min, annealing 59 ^{o}C 0.45 min, extention of 72 ^{o}C 1 min, for 35 cycle, and final extention of 72 ^{o}C 10 min. PCR products were automated sequencing.

5. MHC Haplotypes Identification by Using Single Strand Conformation Polymorphism (SSCP) Method

DNA samples were amplified by using forward and reverse primers of exon2 of BLβII and BFαI and BFαIV genes (Table 3). Each 20 µl reaction contained 20 ng of DNA, 0.5 µM of each primer, 1.9 mM $MgCl_2$, 0.2 mM of each dNTP and 1 unit of *Taq* DNA polymerase in the supplied buffer (final concentration : 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0, 0.01%(w/v) Tween). PCR amplification of DNA was performed in a PTC-100^{TM} Programmable Thermal Controller using the reaction profile of initial denaturation of 94°C 3 min, denaturation of 94 ^{o}C 0.45 min, annealing 54 ^{o}C 0.45 min, extention of 72 ^{o}C 1 min, for 35 cycle, and final extention of 72 ^{o}C 10 min. Aliquots of the PCR reactions were checked for robustness using 2% agarose gel electrophoresis before proceeding to SSCP analysis and automated sequencing.

One to three microliters of PCR reaction product were denatured at 94 ^{o}C in 3 to 9 µl formamide dye (30 mL formamide, 0.05 g each of xylene cyanol and bromophenol blue, and NaOH 10 mM) for 10 min and immediately chilled on ice to prevent reannealing. The denatured PCR products were electrophoresed for 2 to 2.5 h at 200 V (equivalent to 400 to 500 volt-h) in 12% polyacrylamide, 1x TBE (45 m*M* Tris, 45 m*M* boric acid, 1 m*M* EDTA) gels in a Hoefer miniVe electrophoresis and electrotransfer unit. Runs were routinely made in refrigerator (4 to 5 ^{o}C) (Goto *et al,* 2002). Silver nitrate staining gels were scanned and imaged composed with Adobe Photoshop.

Each group of SSCP pattern was standardized by partial sequencing of BLβII and BF1 and BF2 genes to identify MHC haplotype.

6. Evaluation of genetic diversity of MHC genes of Thai indiginious chicken

Genetic diversity of MHC genes was evaluated by using POPGENE version 1.31 and Nei (1972).

The mean genetic variabilities were calculated for each locus and breed composition. The H_o was calculated by directly determining the frequency of heterozygosis:

$$H_{ol} = \sum_{i \neq j} \frac{N_{lij}}{N}$$

where: N_{1ij} is the number of heterozygous individuals at locus 1; N is the total number of individuals analyzed.

The expected heterozygosity (H_e) was calculated based on the observed allelic frequencies:

$$H_{e1} = 1-\sum_{i=1}^{n} p_{1i}^2$$

where: p_{1i} is the frequency of allele i at locus 1; n is the number of alleles at locus 1.

In order to describe the genetic structure of the population and to quantify the genetic diversity, estimates were made of the Nei's diversity index (Nei, 1973), based on differences between allele frequencies: total genetic diversity (H_T), intrapopulational (H_S) and interpopulational diversity (G_{ST}), calculated as:

$$1 - \frac{H_S}{H_T}$$

7. Estimation of influences of MHC haplotypes on Avian Influenza Virus Disease

Data were statistical analyzed by $\chi 2$ analysis to test significant differences of association of MHC class I and class II gene polymorphisms to avian influenza virus disease resistance in Thai indigenous chicken.

8. Places and Duration

This research was conducted at Kabinburi (KB) Livestock Research and Breeding Center, Prachinburi Province, Tabkwang (TK) Livestock Research and Breeding Center, Saraburi Province, Thapra (TP) Livestock Research and Breeding Center, Khonkaen Province, Suratthani (ST) Livestock Research and Breeding Center, Suratthani Province, belonging to Department of Livestock Development, rural area of central part of Thailand in the area of AIV outbreak, and at Center for Agricultural biotechnology at Kasetsart university (Kamphaengsaen Campus), Nakhonpathom Province between October 2002 to March 2005.

Activity	Duration
- Preparation of chemical material	October – December 2002
- Investigation of suitable MHC haplotypes identification method of Thai indiginious chicken	January – June 2003
- Identification MHC haplotypes of Thai indiginious chicken	July – Decembe 2003
- Evaluation of genetic diversity of MHC genes of Thai indiginious chicken	January – March 2004
- Estimation of influences of MHC haplotypes on Avian Influenza Virus Disease resistant traits in Thai	November 2003 – October 2004

indiginious chicken	
- Estimation of the correlations between MHC haplotypes and Avian Influenza Virus Disease resistant traits in Thai indiginious chicken	November 2003 – October 2004
- Result reporting	December 2004 – March 2005

9. Funding Sources

This research was supported for research fund by Department of Livestock Development, Ministry of Agriculture and Coorperative. Rachathevi, Bangkok, Thailand.

RESULTS AND DISCUSSION

MHC Class II Haplotypes Identification by Using LEI0258 Microsatellite Marker

The association between LEI0258 marker and MHC class II haplotypes were very consistent for the same haplotype from the multiple sources. Sequence information for the region defined by LEI0258 was obtained for nine different alleles of homozygous and heterozygous haplotypes(Figure 12).

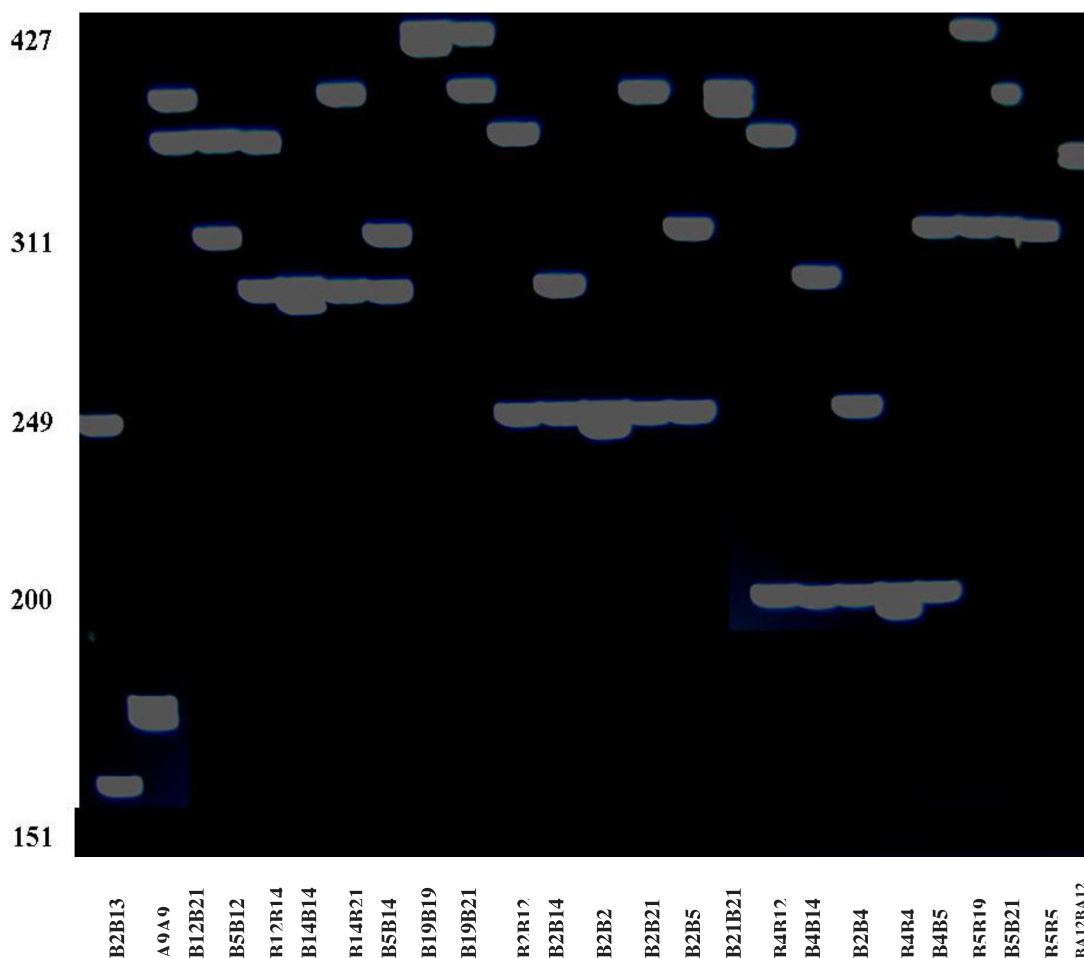


Figure 12 LEI0258 microsatellite markers used to identify MHC class II haplotypes (samples from reference population of Thai indigenous chicken).

After screening MHC class II haplotypes by LEI0258 marker, DNA sample for homozygous group were amplified by primer of BLβ (exon2) and partial sequencing to standardize association between LEI0258 marker band and MHC class II haplotype, and these haplotypes were shown in Table 4.

This marker encompassed two internal repeats whose leghts were 13 bp and 12 bp respectively (Fulton *et al,* 2004). Allele sizes variation ranged from 167 – 427

bp and was due to change the number of both 12 and 13 bp repeated, plused the present or absence of and 8 bp deletion in the unique sequence (Table 4 and Figure 13). This marker will be a useful tool to identify MHC class II haplotypes in chicken, particularly, for the initial development of serological reagent. Eventually, it is easy to work.

CACGCAGCAGAACTTGGTAAGGGAATTCCCTCCCTGGGTTTGTTCTCT TGTTTTTCTCTTTTGGAGGGGGATTTTTTCTATGTCTTCTTTCTTTCCT TCTCTTTCCTTCTTTCTTTCCCTCTTTCTTTCCTTCTTTCTTTCCTTGGA TTTTGAGCCAAAAAAATCACCACAAAATGAGCCTGAATGTTTGCACTG AGGACTGAGCACAGCT

Figure 13 The LEI0258 sequences of each allele were different due to inter-sequence of this marker, primer sequence(red), R13 (blue), R12(green), and 8 bp deletion (pink).

Table 4 MHC class II haplotypes identified by LEI0258 microsatellite marker with there insertion and deletion region

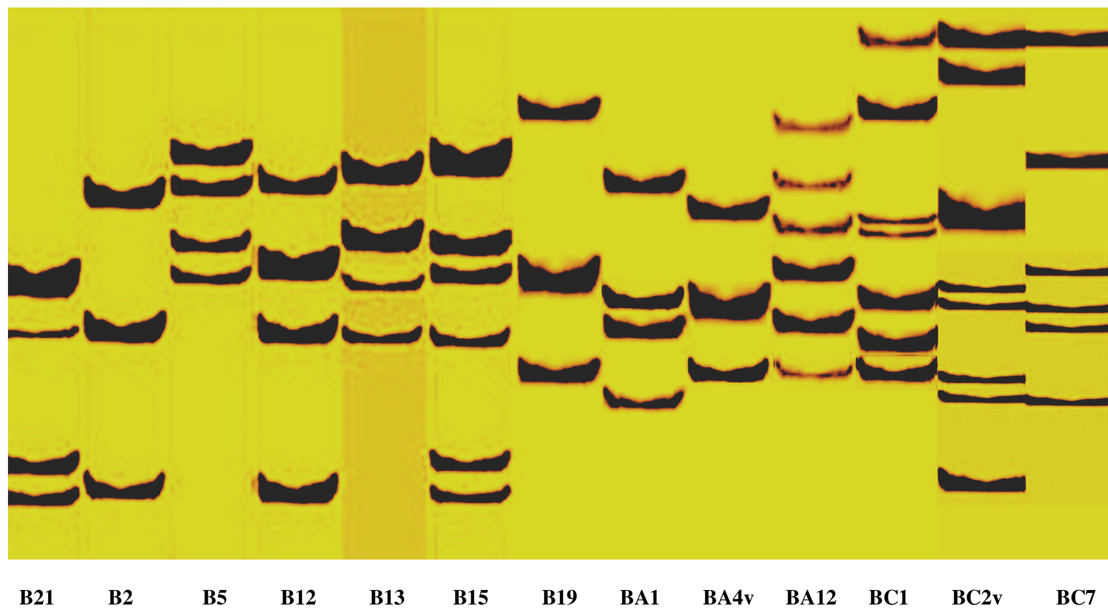
Size	R13[1/]	R12[2/]	D8[3/]	MHC haolotype
167	1	1	+	B13
180	1	1		A9
200	1	3		B4
249	1	7		B2
285	1	10		B14
311	1	12		B5
340	1	14		BA12
370	1	17		B12
388	1	18		B21
427	16	5		B19

[1/] Number of insertion 13 bp repeat
[2/] Number of insertion 12 bp repeat
[3/] Apparentness of deletion of 8 bp repeat

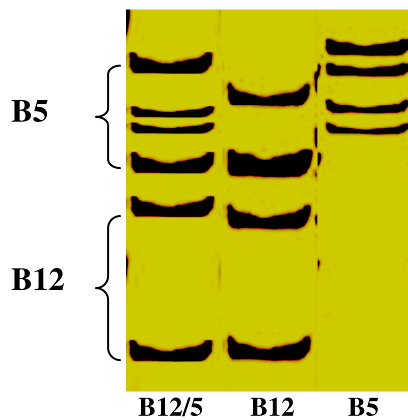
BA12 haplotype sometimes had the same size of LEI0258 as B21 haplotype, 388 bp, this problem could be solved by using CAJF01 marker, compared with BLβII SSCP patterns, sequencing method, and using frequency of event to make decision. This marker is outside LEI0258 marker (Fulton *et al,* 2004). LEI0258 alleles were very variable, but were consistent within a haplotype. This marker could be used to define MHC class II haplotypes in a quick reliable and consistent manner.

MHC Haplotypes Identification by Using Single Strand Conformation Polymorphism (SSCP) Method

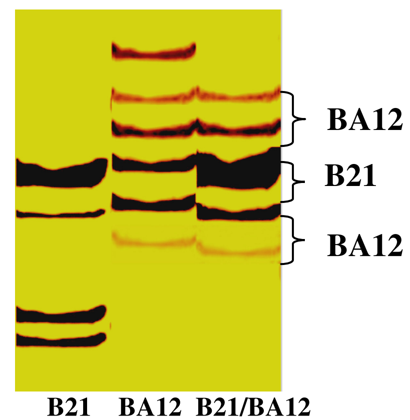
The SSCP typing was used to type MHC class I and class II haplotypes in Thai indigenous chicken with unknown haplotype. At first, MHC class I SSCP patterns were compared the research of Goto *et al* (2002) (Figure 14). Some patterns were similar to Goto's results. The patterns that were similar to Goto's, they were homozygous haplotypes. For these five strains of chicken, there was one group of full pedigree. They could be identified more easily after compared with full pedigree patterns. By the reason of specific BFαI gene primers (Goto *et al*, 2002), these primers particularly specific to SSCP method. There was less change in pattern. In addition, these primers were designed to be able to detect individual SSCP pattern, because source of them was from Goto'research that obtained to develop easy and safe method to detect MHC haplotype. Major group of them was heterozygous. Only homozygous patterns were standardized by partial sequencing of BF1 (αI) to correct haplotypes, because some haplotype are similar sequences.



A



B

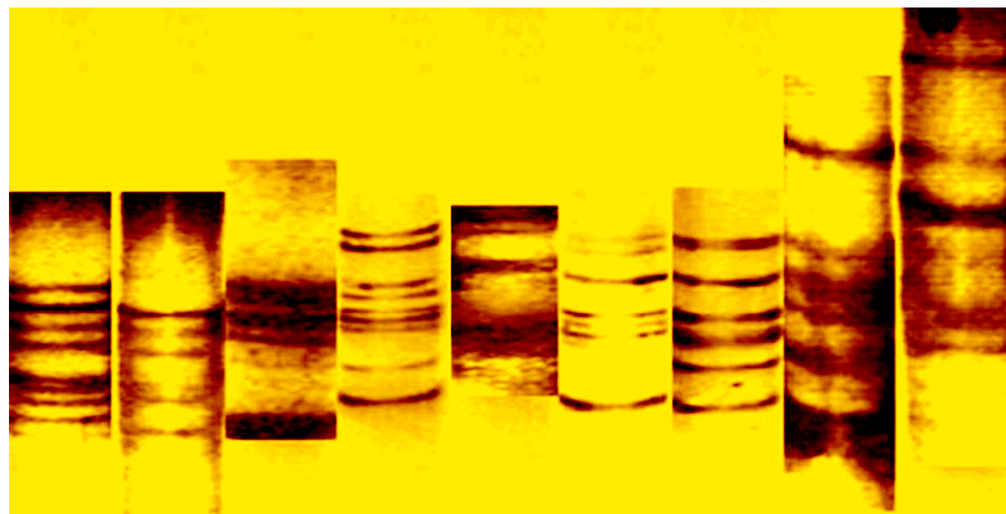


C

Figure 14 SSCP pattern for MHC class I haplotypes. (A) homozygous haplotypes, (B) and (C) heterozygous haplotypes (samples from reference population of Thai indigenous chicken). Some haplotype was only one homozygous sample (B15, B19, and BC2v).

MHC class II patterns were compared to the result of LEI0258 marker and result of SSCP method from the research of Goto *et al* (2002). The results of sequencing and compared with sequences in Genbank homozygous haplotypes by minus of heterozygous results from result of LEI0258 (Figure 15). The LEI0258 marker almost clearly represented homozygous and heterozygous. Furthermore, as the same opinion of MHC class I haplotype identification, other methods are necessary for screening MHC haplotypes, and compared with this method. Some

haplotype that could not clearly separated by LEI0258 alleles, would be confirmed by patterns of MHC class II SSCP. Some patterns were similar to Goto *et al* results. The patterns that were similar to Goto's, they were homozygous haplotypes. Major group of them was heterozygous. Only homozygous patterns were standardized by partial sequencing of BLβ to correct haplotypes, because some haplotype are similar sequences.



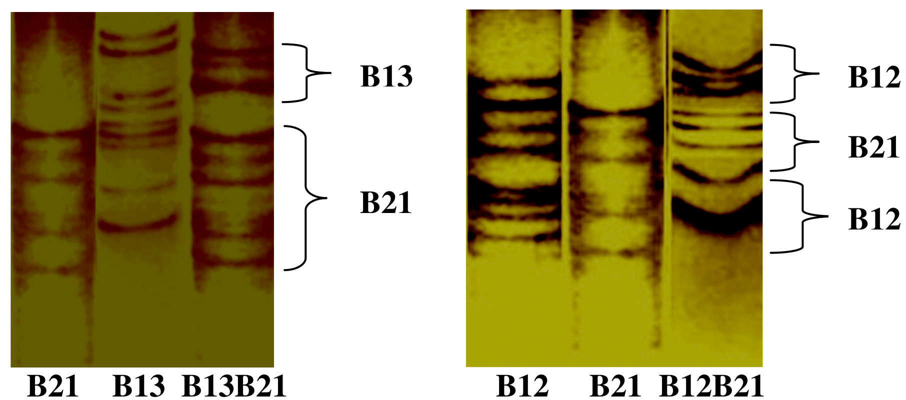
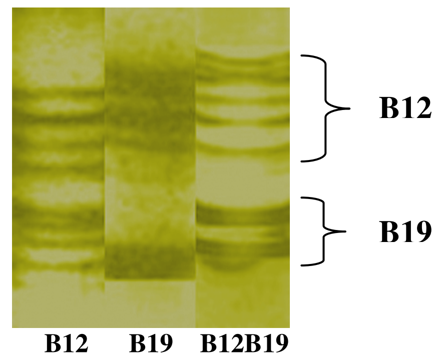


Figure 15 SSCP pattern of MHC class II haplotype (A) Homozygous haplotypes, (B) and (C) heterozygous (samples from reference population of Thai indigenous chicken). Some haplotype was only one sample of homozygote and heterozygote sample (B13, B15, and B13B21).



D

Figure 15 (continued) (D) heterozygous haplotypes

To carry on the work of identification of MHC class II haplotypes by using SSCP method was more easy than working in MHC class I haplotypes, because these haplotype were known haplotype after they identified by LEI0258 typing. However, they had problems about size of PCR products, were over than 200 bp (277 bp), so they were not completely denatured (Miller, *pers. com.*). Size of 277 bp was polymorphic for pathogen binding site of MHC class II of β1 domains. This method might be necessary to develop for suitable work.

The results presented in this research uphold the useful of SSCP into identify MHC genotypes in Thai indigenous chicken. MHC haplotypes in Thai indigenous chicken were various. As samples of SSCP patterns in this research, this method could detect both homozygous and heterozygous at high resolution. In Goto's research paper, this method could detect recombination between MHC class I and class II haplotype with MHC class IV. Such recombinant haplotypes were particularly valuable in commanding the loci within the MHC that are responsible for the strong influences of the B system on immune response and disease resistance in chickens (Goto *et al*., 2002). This method should be developed for identification of MHC haplotype. It was more safty than RFLP method, because RFLP had to use radioactive for sensible results. SSCP method could be used in simple laboratories.

In addition, for easier differences, such as single nucleotide difference between alleles, conditions could be developed (using priming site choice, gel running conditions, temperature, etc.) for optimal separation of the single strands according to the genetic distinctions. From this reason, the differences of single nucleotide mutance detected by SSCP could be developed to single nucleotide polymorphism (SNP) marker. The SNP marker was useful genetic selection program of functional genes. In the more complex systems where there were more loci, such as the BF or BL haplotypes, often the individual bands can still be assigned to each haplotype. The conditions of SSCP method in this research was developed by Goto *et al* (2002). Therefore the patterns may be more complex than might be expected, e.g., band numbers fewer or greater than expected. Because the conditions were not optimized for each allele pair, the migration of single strands with similar conformations may not be fully resolved. With these qualifications with careful attention to detail, SSCP can be used to define B haplotypes. B genotyping by SSCP patterns should become

widely adopted, it was likely that it would be difficult to relate genotypes defined in different laboratories without common standards. Given the sensitivity of SSCP to conditions under which gels were run, it is likely that SSCP patterns identified in different laboratories for the same B haplotype might appear some different. For some objectives it might not be necessary to assign standard haplotype designations, but it certainly would be useful to have a means for forming at least experimental standard B haplotype designations. SSCP might be adapted for genotyping additional polymorphic B loci, such as the TAP loci (Wain, 1997) as well.

Wider application of SSCP assays might be an efficient means of defining allelic unstableness at polymorphic loci within recombinant haplotypes as efforts to understand the strong role of the B system in disease resistance continue. This method can be use easily in simple laboratories. It should be developed to work better in the future.

MHC Haplotypes in Thai Indigenous Chicken

MHC Class I Haplotypes in Thai Indigenous Chicken

The MHC class I haplotypes were identified in Thai indigenous chicken represented 17 haplotypes (some haplotypes were concealed in heterozygous with the other haplotypes) composed of 7 homozygous haplotypes and 24 heterozygous haplotypes (Table 5).

Table 5 MHC class I haplotypes of Thai indigenous chicken

Haplotypes[1/]		Accession no.[2/]	Number of chicken	Percentage of herd
A9	Homozygous	DQ011916	16	6.35
	Heterozygous		45	17.86
B12	Homozygous	DQ011917	10	3.97
	Heterozygous		6	2.38
B13	Homozygous	DQ011918	14	5.56
	Heterozygous		16	6.35
B14	Heterozygous		19	7.54
B17	Homozygous	DQ011919	1	0.40
	Heterozygous		3	1.19
B18	Heterozygous		2	0.79
B19	Heterozygous		2	0.79
B2	Homozygous	DQ011920	6	2.38
B21	Homozygous	DQ011921	33	13.10
	Heterozygous		16	6.35
B5	Homozygous	DQ011922	5	1.98
	Heterozygous		9	3.57
BA1	Homozygous	DQ011923	2	0.79
BA12	Homozygous	DQ011924	1	0.40
	Heterozygous		5	1.98
BA4v	Homozygous	DQ011925	3	1.19
BC1	Homozygous	DQ011926	36	14.29
BC7	Homozygous	DQ011927	2	0.79

[1/] A9, B14, B17, and B18 homozygous and heterozygous haplotypes were identified by sequencing method.
[2/] Accession no. were conceded by Genbank (samples from reference population of Thai indigenous chicken).

These results were identified by SSCP method. Each group of SSCP pattern was standardized by sequencing of exon2 of BF gene and compared with sequences in Genbank homozygous haplotypes by minus of heterozygous results from full pedigree SSCP (Table 6, App. Table 1). Some haplotype was not in homozygous haplotypes. They were compared to Goto *et al* (2002). Furthermore, other methods are necessary for screening MHC haplotypes, and compared with this method. For example,

serological typing (Briles, 1987) and BG RFLP (Miller *et al,* 1988) are effective to MHC haplotype typing. Serological reagents were produced from monoclonal antibody. First, serological reagents have been developed and used in Leghorn chickens. They have been elucidated with respect to BF (classI MHC) and BLβ (classII MHC heavy chain) alleles (Guillemot *et al,* 1988; Kaufman *et al,* 1992). However, serological reagents developed to type standard MHC haplotypes in Leghorn chickens give ambiguous reactions when used outside the line in which they were produced (Livant *et al,* 2001). Standard haplotype and known haplotype are very necessary for this method. Serological reagents must be take 2-3 generations to develop. Thus, in Thai indigenous chicken MHC haplotypes, unknown haplotype, should not used this method. Following this study, MHC haplotypes are known, serological reagents can be developed, this method can be used to type MHC haplotypes. It will be the most effective method, because easily and cheaply used. BG RFLP (Miller *et al,* 1988) are effective to MHC haplotype typing. BG RFLP was used to type MHC haplotypes extensively. Because the BG gene family is large and highly polymorphic, genetic differences in the BG region are easy to detect by Southern hybridization (Goto *et al,* 2002). Restriction fragments produced with any of several restriction endonucleases provide patterns correlated with individual B haplotypes (Miller *et al*, 1988; Uni *et al*, 1993; Li *et al*, 1997; Yonash *et al*, 2000).

Table 6 Summary of nucleotide data of MHC class I haplotypes of Thai indigenous chickens.

Haplotype (identity)[1/]	Reference, GenBank Accession no.
A1(92)	Li *et al* (1999), AF021963
A9(100)	Li *et al* (1999), AF026916
B12(100)	Kaufman,J. (1995), Z54325
B13(98)	Hunt and Fulton(1998), AF013494
B17(95)	Hunt and Fulton(1998), AF013495
B21(100)	Hunt and Fulton(1998), AY234769
B5(96)	Hunt and Fulton(1998), AF013491
BA12(96)	Brigatti *et al* (2002), AF483195
BA4v(96)	Li *et al* (1999), U88299
BC1(96)	Livant *et al* (2001), AF342825
BC7(88)	Livant *et al* (2001), AF094778

[1/] Number in parenthesis mean identity percentage of nucleotide sequences between Thai indigenous chicken MHC haplotypes and standard MHC haplotypes in GenBank (alignment comparing of nucleotide sequences).

BC1 and B21 haplotypes were the highest percentage in herd. The BC1 haplotype (MHC class I haplotype from broiler chicken) had nucleotide sequences similar to B6 of standard haplotype. They were different of one amino acid in pathogen binding site of αI domain (Livant *et al,* 2001). The B21 haplotype was recommended as the strongest disease resistant traits, especially, recombined with B14 haplotype of MHC class II genes. This recombination was the strongest disease resistant traits (Juul-Medsen, *pers. com*). There were 5.56 percentages of homozygous

B13 in Thai indigenous chicken recommended susceptible to all pathogens, considered to recombination with MHC class II. In these populations, there were no B13 haplotype of MHC class II (not true susceptible). Referred the study of Dalgaard *et al* (2003) detecting transcription of 1.9 kb BFI and 1.5 kb BFIV. In BW1, B130, and B21, the two transcripts were almost equally expressed. In B2, B6, B12, and B19, the ratio between the two transcripts was 4:1, with the 1.9 kb transcript having the strongest expression. In B14 and B15, the 1.5 kb transcript was undetectable and the 1.9 kb transcript appeared to be exclusively expressed. Thus, haplotypes considered to have and MHC-determined resistance to Marek's disease (MD) had the highest relative amount of the 1.5 kb transcript, whereas haplotypes considered to be MD-susceptible had the lowest. Animals of haplotype B21, BW1, and B19 were infected with MDV. In 9 weeks post-infection, the relative amount of the 1.5 kb transcript in BW1 and B21 haplotype was shown to be significantly higher than that in B19. Interestingly, in infected BW1 and B21 animals, the relative amount of the 1.5 kb transcript was also significantly higher than that in healthy MHC-matched controls. In B19, no differences were detected between uninfected and infected animals.

In this study, there are many groups of animals resisted to pathogens referred to many researches in the past. In future, following experiment in progeny, challenge with pathogen, they will be resistant to pathogen. Thai indigenous chickens were high resistant to disease, but they had low production traits. Thus, the first priority of genetic improvement was to improve production traits. Production traits have negative correlations with disease resistant traits. Selection pressure on commercially important traits under stressful production settings also may increase the incidence of disease. Disease also impairs genetic improvement in production traits because efficiency of selection is reduced (Rothschild, 1989).

This research was conducted to designate the Thai indigenous chicken MHC haplotypes is provisional. This work is in progress within the chicken MHC research community to standardize the nomenclature of the chicken MHC, as the haplotypes initially defined by serological and DNA bases studies in Leghorns are defined on the molecular level. The future works of molecular characterization of Thai indigenous MHC haplotypes, added with identification of novel haplotypes in these chickens allows inclusion of these MHC haplotypes in this process and their eventual incorporation into the standard nomenclature, providing a more complete picture of the diversity of chicken MHC.

MHC Class II Haplotypes in Thai Indigenous Chicken

The MHC class II haplotypes were identified in Thai indigenous chicken represented 9 haplotypes (some haplotypes were concealed in heterozygous with the other haplotypes) composed of 8 homozygous haplotypes and 16 heterozygous haplotypes (Table 7)

Table 7 MHC class II haplotypes of Thai indigenous chicken

Haplotypes.[1/]		Accession no.[2/]	Number of chicken	Percentage of herd
A9	homozygous	AY973596	4	1.59
B12	homozygous	AY973591	13	5.16
	heterozygous		42	16.67
B14	homozygous	AY993997	6	2.38
	heterozygous		32	12.70
B19	homozygous	AY973592	13	5.16
	heterozygous		8	3.17
B2	homozygous		7	2.78
	heterozygous		42	16.67
B21	homozygous	AY973590	52	20.63
	heterozygous		20	7.94
B5	homozygous		5	1.98
	heterozygous		1	0.40
BA12	homozygous	AY973602	7	2.78

[1/] Some haplotype was identified by sequencing method.
[2/] Accession no. were conceded by Genbank (samples from reference population of Thai indigenous chicken).

These results were identified by LEI0258 marker and SSCP method. Each group of LEI0258 band and SSCP pattern was standardized by sequencing of exon2 of BLβ gene and compared with sequences in Genbank homozygous haplotypes by minus of heterozygous results from LEI0258 (Table 8, App. Table 2). LEI0258 marker almost clearly represented homozygous and heterozygous. Furthermore, as the same opinion of MHC class I haplotype identification, other methods are necessary for screening MHC haplotypes, and compared with this method.

Table 8 Summary of nucleotide data of MHC class II haplotypes of Thai indigenous chickens.

Haplotype (identity)[1/]	Reference, GenBank Accession no.
A9(95)	Li *et al* (1999), AF026562
B12(93)	Guillemot *et al* (1988), AL023516
B14(87)	Jacob *et al* (2000), AJ248579
B19(95)	Jacob *et al* (2000), AJ248584
B2(90)	Jacob *et al* (2000), AJ248572
B21(95)	Jacob *et al* (2000), AJ248585
B5(89)	Pharr *et al* (1998), U91537
BA12(98)	Zheng *et al*(1999), AF036616

[1/] Number in parenthesis mean identity percentage of nucleotide sequences between Thai indigenous chicken MHC haplotypes and standard MHC haplotypes in GenBank (alignment comparing of nucleotide sequences).

The B21 haplotype was the highest percentage in reference population of Thai indigenous chicken. The B21 haplotype was recommended as the strongest disease resistant traits. There was only one B13 haplotype in Thai indigenous chicken recommended susceptible to all pathogens, Two loci of BLβ (BLβI and BLβII) These two genes differ very little from each other in nucleotide sequence, and BLβII genes are expressed at high levels in B lymphocytes (Zheng *et al*.,1999; Zoorob *et al*.,1993). In this study, full region of exon2 of BLβ1 was amplified to test polymorphism, by the reason of this region produced β1 domain of MHC class II protein which was the pathogen binding site and they were polymorphic for each haplotype related to disease resistance (Data were shown in Appendix Table 2).

In this study, there are many groups of animals resisted to pathogens referred to many researches in the past. In future, following experiment in progeny, challenge with pathogen, they will be resistant to pathogen. Thai indigenous chickens were high resistant to disease, because they were selected disease resistant traits by natural selection in many generation.

This research was conducted to designate the Thai indigenous chicken MHC haplotypes is provisional. This work is in progress within the chicken MHC research community to standardize the nomenclature of the chicken MHC, as the haplotypes initially defined by serological and DNA bases studies in Leghorns are defined on the molecular level. The future works of molecular characterization of Thai indigenous MHC haplotypes, added with identification of novel haplotypes in these chickens allows inclusion of these MHC haplotypes in this process and their eventual incorporation into the standard nomenclature, providing a more complete picture of the diversity of chicken MHC.

Genetic Diversity of MHC Haplotypes in Thai Indigenous Chicken

Genetic Diversity of MHC Class I Haplotypes in Thai Indigenous Chicken

Seventeen MHC class I haplotypes were identified in Thai indigenous chickens (Table 9).

Table 9 MHC class I Haplotypes frequencies of Thai indigenous chicken

Haplotypes[1/]	Strain[2/]					
	LHK(76)	PHD(80)	CH(30)	ND(28)	KL(38)	Average
A9	0.1513	0.1313	0.2000	0.1607	0.1579	0.1528
B12	0.0592	0.1187	0.0000	0.0000	0.0263	0.0595
B13	0.1053	0.0750	0.1000	0.1071	0.0526	0.0873
B14	0.0461	0.0875	0.0333	0.0357	0.0000	0.0496
B17	0.0132	0.0000	0.0000	0.0000	0.0395	0.0099
B18	0.0263	0.0813	0.0000	0.0179	0.0263	0.0397
B19	0.0066	0.0000	0.0000	0.0000	0.0132	0.0040
B2	0.0395	0.0125	0.0000	0.0000	0.0526	0.0238
B21	0.1645	0.2188	0.0667	0.1964	0.1842	0.1766
B5	0.0263	0.0500	0.0500	0.0893	0.1184	0.0575
BA1	0.1032	0.0000	0.0000	0.0000	0.0263	0.0079
BA12	0.0526	0.0750	0.0167	0.0357	0.1316	0.0655
BA4v	0.0329	0.0000	0.0000	0.0000	0.0263	0.0139
BC1	0.2303	0.1250	0.5333	0.3214	0.1316	0.2282
BC7	0.0263	0.0125	0.0000	0.0000	0.0132	0.0139
Homozygotes	0.6184	0.4625	0.4000	0.3571	0.6053	0.5119
Heterozygotes	0.3816	0.5375	0.6000	0.6429	0.3947	0.4881

[1/] These haplotypes had accession no. conceded by Genbank of DQ011916-DQ011927 (samples from reference population).
[2/] Numbers in parenthesis were number of chicken.

Haplotype BC1, B21, and A9 were predominant in all strains. The BC1 haplotype was found by Livant *et al.* (2001) in commercial broiler chicken line C. The A9 haplotype was found by Li *et al.* (1999) in commercial chicken line A. Both BC1 and A9 haplotypes had nearly similar nucleotide sequences to the standard B12 haplotype which was identified in White Leghorn chicken. The strongest disease resistant haplotype which was identified in White Leghorn chicken was the standard B21 haplotype (Bacon, 1987). By this reason, Thai indigenous chicken were related to disease resistant traits because they had high frequencies of B21 haplotype. This haplotype was high frequency in PHD chicken population. This reason was by the effect of genetic selection of production and disease resistant traits in many generations of Livestock Research and Breeding Center of Department of Livestock Development. Frequencies of homozygotes were significantly higher in LHK strain and KL strains than in other strains ($P<0.05$), because samples of these two strains were collected from Kabinburi Livestock Research and Breeding Center, they were

genetically selected for high production traits and disease resistant traits in many generations. Frequencies of homozygotes were lower in the CH and ND strains than in other strains ($P<0.05$).

Diversity measures were calculated on the basis of allele frequencies. Table 10 shows the number of alleles per locus and mean heterozygosity for each population.

Table 10 Number of alleles per locus and mean hetrozygosity values obtained from five strains of Thai indigenous chicken population.

Strain	No. of alleles per locus	Mean heterozygosity Direct count
LHK	16.0	0.3816
PHD	13.0	0.5375
CH	7.0	0.6000
ND	9.0	0.6429
KL	14.0	0.3947
Total	17.0	0.4881

The LHK, PHD, and KL strains showed the high allele number., whereas CH strain showed the lowest allele number. Oppositely, LHK, PHD, and KL strains had low heterozygosity, because these strains were raised selected for production traits for along time. However, they had various characteristics of color traits. The LHK and PHD strains were popularly raised in every part of Thailand as fighting chicken. This sport helped these two strains to successfully breed. They were the most divergent and cultural heritage for Thai people. They were the main population of Thai indigenous chicken, and also KL strain, they were raised and were improved genetics in many Center of Department of Livestock Development. The CH strain was not popularly raised in rural area. By the reason of a little number population which founder effects and genetic drift had contributed to the loss of variation or they were concealed in crossbred population.

These haplotypes were found in both homozygotes and hetrozygotes. Haplotype BC1, B21, and A9 were predominant in all strains. BC1 haplotype was found by Livant *et al* (2001) in commercial broiler chicken line C. A9 haplotype was found by Li *et al* (1999) in commercial chicken line A. These two haplotypes had nearly similar nucleotide sequences of BFα1 and nearly similar to standard B12 haplotype of White Leghorn chicken. However, they were recommended as different haplotypes. Standard B21 haplotype was identified in White Leghorn chicken. It was recommended as the strongest disease resistant haplotype (Bacon, 1987; Juul-Medsen, *pers. com.*) By this reason, Thai indigenous chicken were related to disease resistant traits because they had high frequencies of B21 haplotype. This haplotype was high frequency in PHD chicken population. This reason was by the effect of genetic selection of production and disease resistant traits in many generation of Livestock Research and Breeding Center of Department of Livestock Development (DLD). Frequencies of homozygotes were highest in LHK strain and KL stain, because samples of these two strains were collected from Kabinburi Livestock Research and

Breeding Center, they were genetic selected for high production traits and disease resistant traits in many generations. Frequencies of heterozygotes were highest in CH and ND strains, because both of them were raised and improved genetics less than 5 years. Frequencies of homozygotes differed significantly among strains ($P<0.05$), highly in LHK, KL strains, medium in PHD strain, and less in CH and ND strains by the reasons of genetic selection and the aims of raising.

From Table 10, CH strain had the lowest of number of alleles per locus and mean expected heterozygosity. The CH strain could be considered as only one typical population in Thapra Livestock Research and Breeding Center. They were least raised in rural area. The other reason was a lower effect on hetrozygocity due to the loss of low frequency alleles.

In general, mean of heterozygosity was observed in the different strains. The CH strain almost showed deviation from equilibrium in MHC class I locus. They were bought from rural area to be raise and improve genetics in Thapra Livestock Research and Breeding Center less than five years. The CH strain was a small herd and unstable herd. At the first time, the first priority of genetic improvement was color traits and morphological traits, so the color and morphological traits were stable before the other traits, especially, disease resistant traits. In this case, they were almost departured from equilibrium, eventhough, they were suggested as an homogenous population. This might be due to the fact that this herd was bred in the same group in northeastern part of Thailand. However, good breeding plan could protect them from losing heterozygosity and endanger.

Ds genetic distances ranged from 0.053 to 0.524 (Table 11). Nei's genetic identity ranged from 0.592 to 0.949. Considering genetic distances and genetic identities, the closest strains were ND, LHK, and CH strains. The first priority of selection in rural area is color. They were raised together in rural area every part of Thailand for home consumption and fighting sport. They were improved genetics in the same traits together. In the Livestock Research and Breeding Center of Department of Livestock Development, they were raised and improved genetics in the same objectives for production and disease resistant traits. The most distant strains were PHD and CH strains. They had different feather color. The CH strain had white color. Oppositely, the main color of PHD was black. The CH strain was selected for white color and morphology as the first priority, whereas PHD strain was selected for production and disease resistant traits as the first priority. In addition, there were relatively between feather color traits and disease resistant traits. Thus, selection of feather color traits influenced disease resistant traits and immune responsibility traits. Conclusively, genetic diversity of MHC class I haplotype linked to feather color traits.

Table 11 Ds standard genetic distance matrix obtained from the frequencies of MHC class I haplotypes.

	LHK	PHD	CH	ND	KL
LHK	0.000				
PHD	0.114	0.000			
CH	0.160	0.524	0.000		
ND	0.053	0.212	0.092	0.000	
KL	0.140	0.154	0.455	0.181	0.000

Dendrogram was constructed from the distance and identity matrix using the unweighted pair-group method with arithmetic mean (upgMa) of MHC class I locus. Figure 16 shows the phylogenetic tree obtained from previous method.

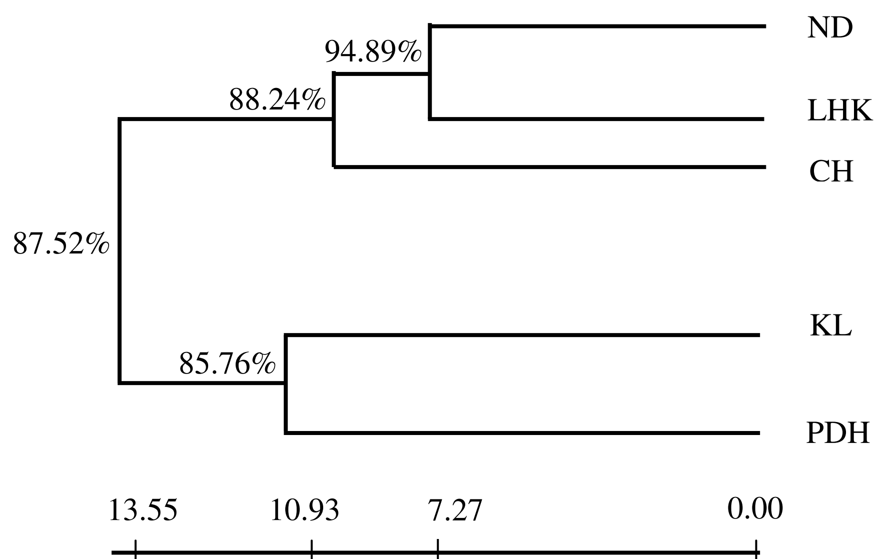


Figure 16 Dendogram showing the genetic relationship of MHC class I among among five strains of Thai indigenous chicken.

Genetic Diversity of MHC Class II Haplotypes in Thai Indigenous Chicken

The MHC class II haplotypes were identified in Thai indigenous chicken kept at Livestock Research and Breeding Center of Department of Livestock Development, composed of LHK, PHD, CH, ND, and KL represented 9 haplotypes (Table 12).

Table 12 MHC class II Haplotypes frequencies of Thai indigenous chicken

Haplotypes[1/]	Strain[2/]					
	LHK(76)	PHD(80)	CH(30)	ND(28)	KL(38)	Average
A9	0.040	0.013	0.000	0.000	0.000	0.016
B12	0.100	0.069	0.217	0.393	0.092	0.135
B14	0.079	0.106	0.167	0.107	0.026	0.093
B19	0.079	0.044	0.067	0.143	0.079	0.073
B2	0.191	0.244	0.100	0.054	0.092	0.167
B21	0.382	0.325	0.150	0.125	0.540	0.331
B5	0.072	0.150	0.283	0.143	0.145	0.149
BA12	0.059	0.050	0.017	0.000	0.026	0.040
Homozygotes	0.474	0.438	0.233	0.357	0.500	0.425
Heterozygotes	0.526	0.563	0.767	0.643	0.447	0.575

[1/] These haplotypes had accession no. conceded by Genbank of AY973590 – AY973605, and AY993991 – AY994017 (samples from reference population).
[2/] Numbers in parenthesis were number of chicken.

Diversity measures were calculated on the basis of allele frequencies. Table 13 shows the mean heterozygosity and number of alleles per locus for each population.

Table 13 Number of alleles per locus and mean hetrozygosity values obtained from five strains of Thai indigenous chicken population.

Strain	No. of alleles per locus	Mean heterozygosity
		Direct count
LHK	8.0	0.5263
PHD	8.0	0.5625
CH	7.0	0.7667
ND	7.0	0.6429
KL	7.0	0.5000
Total	9.0	0.5754

The LHK, and PHD strains showed high allele number and moderated heterozygosity, whereas KL strain showed the lowest heterozygosity. The LHK, PHD strains were popularly raised in every part of Thailand as fighting chicken. This sport helped these two strains be lasting and developing. They were the main population of Thai indigenous chicken. The CH strain were raised as a typical herd at Thapra Livestock Research and Breeding Center, but they were bought from rural area and raised less than five years. They might be concealed in term of crossbred. By this result, these strains of Thai indigenous chicken were high divergent for MHC class II genes that related to immune response and disease resistant traits.

These haplotypes were found in both homozygotes and hetrozygotes. Haplotype B21 was predominant in all strains. Standard B21 haplotype was identified in White Leghorn chicken. It was recommended as the strongest disease resistant

haplotype (Bacon, 1987; Juul-Medsen, *pers. com.*) By this reason, Thai indigenous chicken were related to disease resistant traits because they had high frequencies of B21 haplotype. This haplotype was high frequency in KL, LHK, and PDH chicken population. For KL strain in rural area, they were natural sected for many generations. By observation in rural area, they were very strong and had disease resistant traits. Livestock Research and Breeding Center of DLD bought them from rural area and they were raised to improved genetics for production and disease resistant traits. They were suitable for small holder farm in rural area. For LHK and PHD strain, this reason was by the effect of genetic selection of production and disease resistant traits in many generation of Livestock Research and Breeding Center of DLD. By the other reason, in rural area, farmers raised them as fighting chicken, so they were selected for powerful traits and considered to disease resistant traits, similar reason for KL strain. Frequencies of homozygptes were highest in KL stain for this locus, because samples of this strain were coolected from Kabinburi Livestock Research and Breeding Center, they were genetic selected for high production traits and disease resistant traits in many generations. Frequencies of heterozygotes were highest in CH strain, because they were raised and improved genetics less than 5 years. Frequencies of homozygotes differed significantly among strains ($P<0.05$), highly in LHK, KL strains, medium in PHD strain, and less in CH and ND strains by the reasons of genetic selection and the aims of raising.

In general, heterozygosity was observed in the different strains. The CH strain showed deviation from equilibrium in MHC class II locus. This fact, CH strain had the low alleles number per locus. They were bought from rural area in northeastern part of Thailand to be raise and improved genetics in Thapra Livestock Research and Breeding Center less than five years. It was a small herd, They were not stable in herd. At the first time, the first piority of genetic improvement was color traits and morphological traits, so the color and morphological traits were stable before the other traits, especially, disease resistant traits. In the case of CH strain, they were almost departured from equilibrium, even though, they were suggested as an homogenous population. They might be due to the fact that this herd came from the same origin and were bred in the same group in northeastern part of Thailand. However, good breeding plan could protect them from losing heterozygosity and endanger.

Ds genetic distances ranged from 0.042 to 0.674 (Table 14). Nei's genetic identity ranged from 0.510 to 0.959. Considering genetic distances and genetic identities, the closest strains were LHK and PHD strains being the most divergent. These two strains were the main population of Thai indigenous chicken. They were raised together in rural area every part of Thailand for home consumption and fighting sport. They were improved genetics in the same traits together. In Livestock Research and Breeding Center of DLD, they were raised and improved genetics in the same objectives for production and disease resistant traits. The most distant strains were KL and ND strains. The KL strain was necknaked chicken. They were selected for high production and disease resistant traits, wherease ND strain was selected for feather color and morphology traits as the first priority.

Table 14 Ds standard genetic distance matrix obtained from the frequencies of MHC class II haplotypes.

	LHK	PHD	CH	ND	KL
LHK	0.000				
PHD	0.042	0.000			
CH	0.424	0.290	0.000		
ND	0.578	0.601	0.164	0.000	
KL	0.064	0.130	0.473	0.674	0.000

Dendrogram was constructed from the distance and identity matrix using the unweighted pair-group method with arithmetic mean (upgMa) of MHC class II locus. Figure 17 shows the phylogenetic tree obtained from previous method.

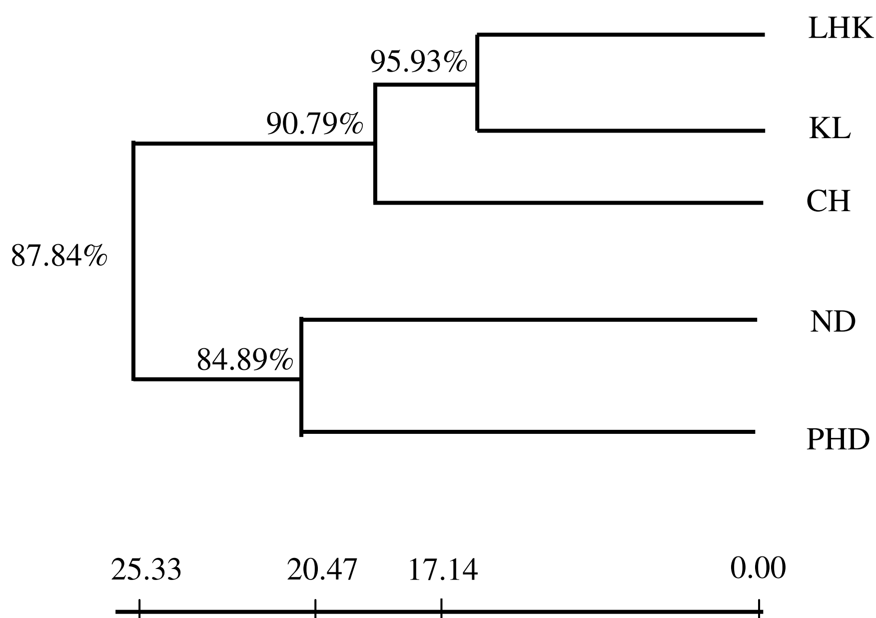


Figure 17 Dendogram showing the genetic relationship of MHC class II among five strains of Thai indigenous chicken.

Influences of MHC Haplotypes on Avian Influenza Virus Disease Traits in Thai Indigenous Chicken

Influences of MHC Class I Haplotypes on Avian Influenza Virus Disease Traits in Thai Indigenous Chicken

MHC class I haplotypes genotyping was used SSCP method based on referenced population. Haplotype which was not in referenced population, was genotyped based on sequencing method. The results represented nine MHC class I haplotypes, A1, B12, B13, B15, B19, B21, B2, B6, and BA12, and included ten heterozygous alleles.

Haplotype of A1 of MHC class I was haplotype in broiler chicken found by Li *et al.*(1999), different from standard haplotypes in White Leghorn chicken. Standard haplotype in White Leghorn chicken preceded with the letter 'B' and followed by numeric. Many scientists suggested that nucleotide sequencing of MHC haplotypes from different breeds were different, because of cross-reaction of serological reagent of White Leghorn using in broiler chicken (Li *et al.,* 1999). B2 – B21 MHC class I haplotypes were identity to standard haplotypes of White Leghorn chicken.

The B haplotypes were identified in Thai indigenous chicken of small holder farms in the rural area of avian influenza virus disease outbreaks in the central part of Thailand represented 28 groups of homozygous and heterozygous haplotypes (Table 15).

Table 15 Association of MHC class I gene polymorphisms to avian influenza virus disease resistant trait

Haplotypes[1/]	Number of chicken[2/]	% survival[3/]
LHK		
A1A1	10	0.00[d]
B12B12	33	0.00[d]
B12B13	21	4.76[d]
B12B21	18	100.00[a]
B13B13	44	0.00[d]
B13B15	19	0.00[d]
B13B2	8	0.00[d]
B13BA12	4	0.00[d]
B15B2	9	77.77[a]
B15B21	26	100.00[a]
B15B5	10	30.00[c]
B19B19	9	22.22[c]
B19B5	10	50.00[b]
B21B21	53	100.00[a]
B21BA12	16	87.50[a]
B2B21	9	100.00[a]

Table 15 (continued)

Haplotypes1/	Number of chicken2/	% survival3/
PHD		
A1A1	18	0.00[d]
B12B12	111	2.70[d]
B12B13	14	7.14[d]
B12B21	8	100.00[a]
B13B13	50	0.00[d]
B13B15	27	0.00[d]
B13B2	10	0.00[d]
B13BA12	4	0.00[d]
B15B2	7	100.00[a]
B15B21	10	100.00[a]
B15B5	6	33.33[c]
B19B19	5	80.00[a]
B19B5	8	50.00[b]
B21B21	121	100.00[a]
B21BA12	16	87.50[a]
B2B21	7	100.00[a]

1/ LHK = Leung-Hahng-Kow chicken, PHD = Pradoo-Hahng-Dam
2/ Data not shown for minor group chicken.
3/ The different letter in same class of MHC means highly significant difference (P<0.01).

The B21 haplotype in MHC class I was the strongest resistant haplotype. The haplotype of B21 survived 100 percent in class I (Table 15). The homozygous B21 haplotype was only found in survived group, so the result showed 100% survived. Homozygous and heterozygous B21 were the majority group of survival chicken. The B12 haplotype was found in dead chicken, recommended as susceptible group. The B12 haplotype in many researches were the same case in as this study. It was susceptible to disease (not all disease) (Bacon, 1987). The heterozygous B12 was higher survival rate than B12 homozygous haplotype. The majority of dead chicken were haplotype B13 and there heterozygotes (100% in mortality rate). Similarly, homozygous B13 haplotype was only found in dead group, so they had 100% mortality rate. In addition, for survival group, B21 haplotype and there heterozygote were the highest survival rate (100% in survival rate), and B2, B6 haplotype were 100% of survival rate, but they were the minority group. The TAP2 gene lied between BF1 and BF2 genes (α genes) on BF/BL region (Bumstead *et al.*, 1994). The TAP2 gene associated with MHC class I on immune response and disease resistant traits. In this maner, TAP2 effected on different levels of MHC class I genes on immune response and disease resistant traits.

In AIV outbreak populations of broilers and layers, the most population was dead. The least survived, because genetic selection by the primary breeder contributed sufficient genetic diversity, because broilers were continueing progress in production

traits. There was less polymorphism in MHC genes. By this way, broiler population had sufficient genetic diversity in disease resistant traits. Broilers and layers were opposite genetic diversity of disease resistant traits, compared with Thai indigenous chicken.

Thai indigenous chicken of small holder farms in the rural area were raised according to natural environment. They were selected by natural selection. The strong chicken were alive and reproductive. The susceptible chickens were culled by nature passed through many generations, dead, and extinct. Further experiment, combination between MHC class I and II affecting on avian influenza virus disease will be apparently detected. The various combinations between showed various levels of disease resistance. There were highly polymorphisms of MHC class I haplotypes in region of BFαI exon2, PBS (pathogen binding site region). This region was specific to pathogen, so MHC haplotypes were highly polymorphism in disease resistant traits (Livant *et al.,* 2001).

By mean of the central role of immunological system, MHC class I presented intracellular antigen to T-cytotoxic after antigen went into host cell by antigen specific receptor, antigens were destroyed. By the same case, reported by Ito(2002). It was reported that virulent avian influenza A viruses produce lethal disease in chickens. Since cell death could be caused by either necrosis or apoptosis, it was investigated the types of cell death that occur in natural hosts, chickens, infected with virulent avian viruses. Using biochemical methods, it was demonstrated that harmful avian influenza viruses induced apoptosis of vascular endothelial cells in liver, kidney, and brain. Viral antigens were also detected in these organs, suggesting that viral replication induced apoptosis in infected chickens. These results indicated that apoptosis did occur in virulent avian influenza virus infection in a natural host, and might contribute to the lethality of the virus. By this way, MHC class I associated with AIV disease in chicken.

After the outbreaks, many of chicken including survived Thai indigenous chicken, were destroyed to eradicate avian influenza virus disease. It is suitable to short term controlling. In long term controlling, it is better to improve disease resistant traits in poultry by using candidate genes of disease resistance as genetic marker associating with conventional breeding. Disease resistant trait improvement can’t process in only conventional breeding according to negative correlation between production traits and disease resistant traits. High intensity of selection of production traits compresses disease resistant traits (Rothschild, 1989). In conventional breeding, step of progeny test, it can’t challenge pathogens to select sire or dam. Therefore, genetic markers of disease resistant genes such as MHC genes, are suitable to improve disease resistant traits.

The MHC class I genes influenced on avian influenza virus disease traits in Thai indigenous chicken. There are polymorphisms of these genes. The MHC genes can be used as genetic markers to improve disease resistant traits in chicken (indigenous chicken, broiler, and layers) for disease prevention strategy. Finally,

poultry industry has high standards in the world for raising healthy livestock and providing consumers with safe products.

Influences of MHC Class II Haplotypes on Avian Influenza Virus Disease Traits in Thai Indigenous Chicken

MHC class II haplotypes genotyping was used LEI0258 marker based on referenced population. Haplotype which was not in referenced population, was genotyped based on sequencing method. The results represented ten MHC class II haplotypes, A9, B12, B13, B14, B19, B21, B2, B4, B5, and B6, and included eighteen of heterozygous alleles. Haplotype of A9 of MHC class II was haplotype in broiler chicken found by Li *et al.* (1999), different from standard haplotypes in White Leghorn chicken.

The MHC haplotypes were identified in Thai indigenous chickens of small holder farms in the rural area of avian influenza outbreaks in the central part of Thailand represented 28 groups of homozygous and heterozygous haplotypes (Table 16).

Table 16 Association of MHC class II gene polymorphisms to avian influenza virus disease resistant trait

Haplotypes[1/]	Number of chicken[2/]	% survival[3/]
LHK		
A9A9	31	90.32[a]
B12B12	39	0.00[c]
B12B13	4	0.00[c]
B12B2	24	66.67[b]
B13B13	71	0.00[c]
B13B2	6	0.00[c]
B14B14	8	100.00[a]
B14B19	4	0.00[c]
B14B2	3	100.00[a]
B19B19	5	0.00[c]
B21B21	74	100.00[a]
B21B4	5	20.00[c]
B2B2	6	66.67[b]
B2B21	10	90.00[a]
B2B5	16	43.75[b]
B4B4	4	50.00[b]
B5B5	3	66.67[b]
PHD		
A9A9	31	90.32[a]
B12B12	92	0.00[c]
B12B13	4	0.00[c]
B12B2	24	66.67[b]
B13B13	71	0.00[d]

Table 16 (continued)

Haplotypes[1/]	Number of chicken[2/]	% survival[3/]
B13B2	6	0.00[d]
B14B14	2	100.00[a]
B14B19	4	0.00[d]
B14B2	3	100.00[a]
B19B19	5	0.00[d]
B21B21	103	100.00[a]
B21B4	5	20.00[d]
B2B2	6	66.67[b]
B2B21	7	85.71[a]
B2B5	16	43.75[c]
B4B4	4	50.00[b]
B5B5	3	66.67[b]

[1/] LHK = Leung-Hahng-Kow chicken, PHD = Pradoo-Hahng-Dam
[2/] Data not shown for minor group chicken.
[3/] The different letter in same class of MHC means highly significant difference (P<0.01).

The B21 haplotype in MHC class II was the strongest resistant haplotype. The B12 haplotype was found in dead chickens, recommended as susceptible group. The B12 haplotype in many researches were the same case in as this study. It was susceptible to disease (not all disease) (Bacon, 1987). In MHC class II haplotypes, similarly to MHC class I, homozygous B21 haplotype chicken survived 100 percents and they were major group, also to their crossbred with other haplotypes were high survival percentage, because of effected expression as co-dominant of this gene. They had the same reason of 100% survived and dead as in MHC class I. The B14 homozygous haplotypes and their heterozygotes were high survival. In addition, A9 haplotype was 90 percents survival rate. The sequences of B21, B14, and A9 haplotype at exon2 of BLβ were almost similar. This region of gene produced protein as pathogen binding site. This region was highly polymorphic and specific to pathogen in each haplotypes. The B13 haplotype were 100 percents of mortality rate, and they were the major group of dead chickens. The same as B19 haplotype, they were 100 percents dead. However, their heterozygous haplotypes were not 100 percent survived, or 100 percent dead, because the effect of co-dominant gene expression.

Further experiment, combination between MHC class I and II affecting on avian influenza will be apparently detected. The various combination among haplotypes showed various levels of disease resistance. The regions of BLβI exon2 of MHC class II haplotypes were specific to pathogen, pathogen binding site, so MHC class II haplotypes were highly polymorphism in disease resistant traits (Livant *et al.,* 2001).

The MHC class II genes associated to avian influenza trait in Thai indigenous chickens. There are polymorphisms of these genes. The MHC genes can be used as genetic markers to improve disease resistant traits in chickens (indigenous chicken, broiler, and layers) for disease prevention. Finally, poultry industry has high standards in the world for raising healthy livestock and providing consumers with safe products.

CONCLUSION

MHC haplotypes identification method

The method for determining MHC class I and II alleles is an alternative to other techniques that has its particular advantages. SSCP (single-stranded conformational polymorphism) assays can be performed in laboratories having access to a PCR thermal cycler, equipment for PAGE and computers supporting commonly used graphics software. The SSCP patterns are detected by silver staining, eliminating the need to handle 32P-labeled nucleic acids. The PCR primer pairs for SSCP described here were developed using well-defined B haplotypes and evaluated with a variety of samples representing inbred lines in which B alleles are fixed, and also various genetic stocks in which B types segregate. They can be applied immediately to produce SSCP patterns to provide information on the MHC genotypes without the need of developing additional reagents. SSCP method canbe used well to identified both MHC class I and class II haplotypes. LEI0258 microsatellite marker can be used clearly to identified MHC class II haplotypes.

MHC haplotype

The MHC class I haplotypes were identified in Thai indigenous chicken represented 17 haplotypes (some haplotypes were concealed in heterozygous with the other haplotypes) composed of 7 homozygous haplotypes and 24 heterozygous haplotypes. The MHC class II haplotypes were identified in Thai indigenous chicken represented 9 haplotypes (some haplotypes were concealed in heterozygous with the other haplotypes) composed of 8 homozygous haplotypes and 16 heterozygous haplotypes.

Genetic diversity of MHC haplotypes

The MHC class I and II haplotypes of Thai indigenous chicken were highly divergent. There were highly genetic diversity of MHC genes in population of DLD and population of small holder farm in rural area of Thailand. By this reason, Thai indigenous chicken were suitable to be base genetic resources of disease traits.

MHC haplotype and Avian Influenza Virus Disease traits

It was found that B21 haplotype, in MHC class I and class II and their heterozygous haplotype, was the strongest disease resistance haplotype. The MHC genes were one system in immunological system pathway, so it is very neccessory to used these genes as genetic markers in genetic selection to improve Avian Influenza Virus disease resistant traits in strategy of disease prevention (does not strategy of disease controllability).

RECOMMENDATION FOR FUTURE WORK

1. Any method of MHC haplotype identification in different laboratories, different population, is neccessory to standardize for accurate results.

2. MHC haplotypes and AIV resistant status of Thai indigenous chicken can be utilized in genetic improvement of disease resistant traits of broiler, layer, and Thai indigenous chicken by using them in breeding plan of these chicken. Finally, poultry industry can produce high production, disease resistance, low cost, and safty chicken. This research results can be obtained to improve animal welfare of poultry industry, healthy farmers, and providing consumers with safty products. From these results, this research will be utilized for chicken meat exportation. Besides of these details, knowledge of the gene may also suggest to improve pharmaceutical or immunological therapies such as DNA vaccines.

3. Avian Influenza Virus Disease is very important to poultry industry economics and human safety life. The other genes that associated with Avian Influenza Virus Disease, especially, specific receptor to AIV. This gene can indicate AIV disease resistant traits in Thai indigenous chicken (true resistance). These characteristics will be very valuable in strategies of disease prevention and disease controllability. In addition, other genes in immunological system pathway, such as T cell, B cell, cytokine, ect. These genes are neccessory to be in research plan for eradication of AIV disease.

4. Furthermore, Thai indigenous chicken of known haplotype can used to develop monoclonal antibodies to identify MHC haplotype in Thai indigenous chicken by serological typing, low price and practical method.

5. In addition, genetic marker developments, such as single nucleotide polymorphism (SNP) of MHC genes were necessary for genetic selection strategy in poultry brreding program.

LITERATURE CITED

Abbas, A.K., A.H. Lichtman, and J.S. Pober. 2000. **Cellular and molecular immunology. 4th ed.** W.B.Saunders company. Pennsylvania. USA. 553 pp.

Anonymous. 2002. Virginia destroys more than 1.4 million stricken fowlVirginia destroys more than 1.4 million stricken fowl. **DVM.** 33: 51.

Bacon, L.D. 1987. Influence of the major histocompatibility complex on disease resistance and productivity. **Poult. Sci.** 66: 802-811.

______ and R.L. Witter. 1994. B haplotype influence on the relative efficacy of Marek's disease Vaccines in commercial chickens. **Poult. Sci.** 73: 481-487.

Benacerraf, B., J. Dausset, and G. Snell. 1980. The 1980 Nobel Prize in Physiology or Medicine NOBELFÖRSAMLINGEN KAROLINSKA INSTITUTET THE NOBEL ASSEMBLY AT THE KAROLINSKA INSTITUTE 10 October 1980. **Available: http://www.nobel.se/medicine/laureates/1980/press.html**, Mar 15,2002.

Bernot, A., R. Zoorob, and C. Auffray. 1994. Linkage of a new member of the lectin supergene family to chicken Mhc genes. **Immunogenetics.** 39:221-229.

Bloom, S.E. and L.D. Bacon. 1985. Linkage of the major histocompatibility (B) complex and the nucleolar organizer in the chicken--assignment to a microchromosome. **J. Hered.** 76: 146-154.

Bourlet, Y., G. Behar, F. Guillemot, N. Frechin, A. Billaut, A. Chausse, R. Zoorob, and C. Auffray. 1988. Isolation of chicken major histocompatibility complex class II (B-L) beta chain sequences: comparison with mammalian beta chains and expression in lymphoid organs. **EMBO J.** 7: 1031-1039.

Briles, C.O., W.H. McGibbon, and M.R. Irwin 1959. Additional alleles affecting red blood cell antigens in chicken. **Genetics** 44: 955-956.

Briles, W.E. 1951. A new blood group in chickens. **Poult. Sci.** 30: 907-908.

______and R.W. Briles. 1982. Identification of haplotypes of the chicken major histocompatibility complex (B). **Immunogenatics** 15: 449-459.

______1987. Genetics and classification of major histocompatibility complex antigens of the chicken. **Poult. Sci**. 66: 776-781.

______, C.O. Briles and J.H. Quisenberry. 1963. The C and D blood group systems in the chicken. **Poult. Sci.** 42: 1096-1103.

______, H.A. Stone, and R.K. Cole. 1977. Marek's disease : effects of B histocompatibility alloalleles in resistant and susceptible chicken lines. **Science** 195: 193-195.

______, N. Bumstead, D.L. Ewert, D.G. Gilmour, J. Gogusev, K. Hala, C. Koch, B.M. Longenecker, A.W. Nordskog, J.R.L. Pink, L.W. Schierman, M. Simonsen, A. Toivanen, O. Vainio, and G. Wick. 1982. Nomenclature for chicken major histocompatibility (B) complex. **Immunogenetics** 15: 441-447.

______, R.M. Goto, C. Auffray and M.M. Miller. 1993. A polymorphic system related to but genetically independent of the chicken major histocompatibility complex. **Immunogenetics** 37: 408-414.

______, W.H.McGibbon, and M.R.Irwin. 1950. On multiple alleles affecting cellular antigens in the chicken. **Genetics** 35: 633-652.

Bronze, M.S. and R.A. Greenfield. 2005. Miscellaneous threats: Highly pathogenic avian influenza, and novel bio-engineered organisms. Available: **http://www.agnr.umd.edu/avianflu/,** Mar 15,2004.

Bumstead, N. and J. Palyga. 1992. A preliminary linkage map of the chicken genome. **Genomics** 13: 690-697.

______, J.R.Young, C.Tregaskes, J.Palyga, and P.P.J.Dunn. 1994. Linkage mapping and partial sequencing of 10 cDNA loci in the chicken. **Available: http://poultry.mph.msu.edu/resources/Conmap/ References.pdf.html**, March 15,2002.

______, R.L. Reece, and J.K.A. Cook. 1993. Genetic differences in susceptibility of chicken lines to infection with infectious bursal disease virus. **Poult. Sci.** 72: 403-410.

Chausse, A.M., F. Coudert, G. Dambrine, F. Guillemot, M.M. Miller, and C. Auffray. 1989. Molecular genotyping of four chicken B-complex haplotypes with B-Lbeta, B-F, and B-G probes. Available: **http://www.poultryscience.org/psa/toc/papers/00/ps00305.pdf.hmtl,** March 15,2002.

Chen, Y., H.S. Lillehoj, C.H. Hsu, S.L. Carpenter, and S.J. Lamont. 1997. Functional characterization of a chicken major histocompatibility complex class II B gene promoter. Available: **http://link.springer.de/link/service/journals/00251/tocs/t7045004.html,** March 15,2002.

Cole, R. K. 1968. Studies on genetic resistance to Marek's disease. **Avian Dis**. 12: 9-28.

Crittenden, L.B., W.E. Briles, and H.A. Stone. 1970. Susceptibility to an avian leukosis sarcoma virus: a close association with an erythrocyte isoantigen. **Science** 169: 1324-1325.

Crone, M., J. Jensenius, and C. Koch. 1981. Evidence for two populations of B-L (Ia-like) molecules encoded by the chicken MHC. **Available: http://www.poultryscience.org/psa/toc/papers/01/ps011109.pdf.html,** March 15,2002.

Department of Livestock development. 2004. Avian influenza virus disease. Available : **http://www.dld.go.th/home/bird_flu/bird_flu1.html**, July 15,2004.

Division of Animal Husbandry. 2003. **Characteristics of Thai indigenous chicken.** Division of Animal Husbandry. Department of Livestock Development. Bangkok. Thailand. 55 pp.

Dominguez, Steglich.M., C. Auffray, and M. Schmid. 1991. Linkage of the chicken MHC to the nucleolus organizer region visualized using non-isotopic in situ hybridization. **J. Hered.** 82: 503-505.

Dunnington, E.A., R.W. Briles, W.E. Briles, W.B. Gross, and P.B. Siegel. 1984. Allelic frequencies in eight alloantigen systems of chickens selected for high and low antibody response to sheep red blood cells. **Poult.Sci**. 63: 1470-1472.

Emara, M. G., H. Kim, J. Zhu, R. R. Lapierre, N. Lakshmanan, H. S. Lillehoj. 2002. Genetic diversity at the major histocompatibility complex (B) and microsatellite loci in three commercial broiler pure lines. **Poult. Sci.** 81:1609-1617.

Ewald, S. J. and E. J. Livant. 2004. Distinctive polymorphism of chicken B-FI (major histocompatibility complex class I) molecules. **Poult. Sci.** 83: 600-605.

Ewert, D.L., D.G. Gilmour, W.E. Briles, and M.D. Cooper. 1980. Genetics of Ia-like alloantigens in chickens and linkage with B major histocompatibility complex. **Available: http://www.bios.niu.edu/briles/cv.html**, March 15,2002.

FAO. 2004. Avian influenza virus disease. **Available : http://www.fao.org/ag/againfo/subjects/en/health/diseases-cards/special_avian.html**, July 15,2004.

Frangoulis, B., I. Park, F. Guillemot, V. Severac, C. Auffray, and R. Zoorob. 1999. Identification of the Tapasin gene in the chicken major histocompatibility complex. **Available: http://link.springer-ny.com/link/service /journals /00251/tocs/t9049004.html**, March 15,2002.

Francis, C. Y., B. Timothy., R. Yang, Y. Zhihong, and M. X. Judy. 1999. **POPGENE VERSION 1.31.** University of Alberta. USA. 29 pp.

Fulton, J.E., E.L. Thacker, L.D. Bacon, and H.D. Hunt. 1995. Functional analysis of avian class I (BFIV) Glycoproteins by epitope tagging and mutagenesis in vitro. **Euro. J. Immunol.** 25: 2069-2076.

Fulton, J.E., H. Juul-Madsen, C.M. Ashwell, A.M. McCarron, and R.L. Taylor. 2004. Molecular genotype identification of the chicken major histocompatibility complex. **29th International Conference on Animal Genetics ISAG2004/TOKYO**. September 11 – 16, 2004. Meiji University, Surugadai Campus. Japan.

Goto, R.M., C.G. Miyada, S. Young, R.B. Wallace, H. Abplanalp, S.E. Bloom, W.E. Briles, and M.M. Miller. 1988. Isolation of a cDNA clone from the B-G subregion of the chicken histocompatibility (B) complex. Available: **http://www.bios.niu.edu/briles/cv.html**, March 15,2002.

______, M. Afanassieff, J. Ha, G.M. Iglesias, S.J. Ewald, W.E. Briles, and M.M. Miller. 2002. DNA-based major histocompatibility complex *B* typing. **Poult. Sci.** 81: 1832-1841.

Guillemot, F., A. Billault, O. Pourquie, G. Behar, A.M. Chausse, R. Zoorob, G. Kreibich, and C. Auffray. 1988. A molecular map of the chicken major histocompatibility complex : the class II β genes are closely linked to the classI genes and the nucleolar organizer. **EMBO J.** 7: 2775-2785.

______. and C. Auffray. 1989. Molecular biology of the chicken major histocompatibility complex. Available: http://www.gene.ucl.ac.uk/users/hester/thesis.html**, March 15,2002.**

Hala, K., M. Vilhelmova, and J. Hartmanova. 1976. Probable crossing-over in the B blood group system of chickens. Available: **http://www.poultryscience.org/psa/toc/papers/01/ps011109.pdf.html**, March 15,2002.

Hansen, M.P., G.R.J. Law, J.N. Van Zandt. 1967. Differences in susceptibility to Marek's disease in chickens carrying two different B locus blood group alleles. **Poult. Sci**. 46: 1268.(Abstr.)

Hanson, J.deG. 2004. Avian Influenza: What It Is and How to Protect Against Its Establishment. **Available: http://www.wvu.edu/~agexten/poultry/avianflu.pdf**, December 15,2004.

Heller, E.D., Z. Uni, and L.D. Bacon. 1991. Serological evidence for major histocompatibility complex (B complex) antigens in broilers selected for humoral immune response. **Poult. Sci.** 70: 726-732.

Hepkema, B.G., A. Van der Poel, M.C. Grosfeld Stulemeyer, and E.J. Hensen. 1991. Biochemical identification of B-F and B-G allelic variants of the chicken major histocompatibility complex. **Available: http://www.labs.roslin.ac.uk/jwilliam/refs.html**, March 15,2002.

Hunt, H.D. and J.E. Fulton. 1998. Analysis of polymorphisms in the major expressed class I locus (B-FIV) of the chicken. **Immunogenetics.** 47: 456-467.

_____, G.T. Pharr, and L.D. Bacon. 1994. Molecular analysis reveals MHC classI intra-locus recombination in the chicken. **Available: http://iubio.bio.indiana.edu/R175522-182101-/news/bionet/journals/contents/9412.newsm.html**, March 15,2002.

Ito, T. 2000. Interspecies transmission and receptor recognition of influenza A viruses. **Microb. Immunol**. 44: 423-430.

Jacob, J.P., S. Milne, S. Beck, and J. Kaufman. 2000. The major and a minor classII β-chain (B-LB) gene flank the Tapasin gene in the B-F/B-L region of the chicken major histocompatibility complex. **Available: http://mapmgr.roswellpark.org/byJournal.html**, March 15,2002.

Jaffe, W.P. and E.M. McDermid. 1962. Blood groups and splenomegaly in chick embryos. **Science**. 137: 984-985.

Johnson, L.W. and S.A. Edgar. 1984. Ea-A and Ea-E cellular antigen genes in Leghorn lines resistant and susceptible to acute cecal coccidiosis. **Poult. Sci**. 63: 1695-1704.

Juul, M.H.R., R. Zoorob, C. Auffray, K. Skjodt, and J.E. Hedemand. 1997. New chicken Rfp-Y haplotypes on the basis of MHC class II RFLP and MLC analyses. **Available: http://www.agrsci.dk/arspublikationer/publiresultforfatuk.asp?forfatter=1260.html,** March 15,2002.

Kaufman, J., H. Volk, and H.J. Wallny. 1995. A "Minimal essential Mhc" and an "Unrecognised Mhc": Two extremes in selection for polymorphism. Available: **http://www.poultryscience.org/psa/toc/papers/00/ps001736.pdf.html**, March 15,2002.

______, N. Bumstead, M. Miller, P. Reigert, and J. Salomonsen. 1993. The chicken class II a gene is located outside of the B complex. In: Davison, T., N. Bumstead and P. Kaiser. (Eds.), **Advances in Avian Immunology Research. Carfax**. pp. 119-127.

______, R. Andersen, D. Avila, J. Engberg, J. Lambris, J. Salamonsen, K. Welinder, and K. Skjodt. 1992. Different features of the MHC classI heterodimer had evolved at different rates. Chicken B-F and β_2-microglobulin sequences reveal invariant surface residues. **J. Immunol.** 148: 1532-1546.

______, S. Milne, T.W.F. Gobel, B.A. Walker, J.P. Jacob, C. Auffray, R. Zoorob, and S. Beck. 1999. The chicken B locus is a minimal essential major histocompatibility complex. **Nature.** 401: 923-925.

Kendal,F.P., C.R.Madeley, and W.H.Allan. 1971. Antigenic relationships in avian influenza A viruses: identification of two viruses isolated from turkeys in Great Britain during 1969-1970. **J. Gen. Virology**. 13: 95-100.

Kiyotani, K., N. Takei, M. Senoo, S. Takao, K. Otsuki, M. Tsubokura, and T. Yoshida. 1987. Enzymological characteristics of avian influenza A virus neuraminidase. **Micro. Immunol.** 31: 1131-1135.

Koch, G., K. Skjodt, A. Toivanen, and P. Toivanen. 1983. New recombinant within the MHC (B complex) of the chicken. **Available: http://www.poultryscience.org/psa/toc/papers/98/ps981858.pdf.html**, March 15,2002.

Lakshmanan, N., J.S. Gavora, and S.J. Lamont. 1997. Major histocompatibility complex class II DNA polymorphisms in chicken strains selected for Marek's disease resistance and egg production or of egg production alone. **Poult. Sci**. 76: 1517-1523.

Lamont, S.J. 1989. The chicken major histocompatibility complex in disease resistance and poultry breeding. **J. Dairy Sci.** 72: 1328-1333.

Lamont, S.J., B.M. Gerndt, C.M. Warner, and L.D. Bacon. 1990. Analysis of restriction fragment length polymorphisms of the major histocompatibility complex of 15I5-B-congenic chicken lines. **Poult. Sci**. 69: 1195-1203.

Landesman, E., Z. Uni, and E.D. Heller. 1993. Designation by restriction fragment length polymorphism of major histocompatability complex classIV haplotypes in meat-type chickens. **Available: http://www.poultryscience.org/psa/toc/papers/00/ps00305.pdf.html**, March 15,2002.

Lee, M.S., P.C. Chang, J.H. Shien, M.C. Cheng, and H.K. Shieh. 2001. Identification and subtyping of avian influenza viruses by reverse transcription-PCR. **J.Viro.Meth**. 97: 13-22.

LePage, K.T., W.E. Briles, F. Kopti and R.L. Taylor, Jr. 2000. Non-major histocompatibility complex effects on the fate of Rous sarcomas. **Poult. Sci.** 79: 343-348.

Li, L., L.W. Johnson, and S.J. Ewald. 1997. Molecular characterization of major histocompatibility complex (B) haplotypes in broiler chickens. **Anim. Gen.** 28: 258-267.

______, E.J. Livant, and S.J. Ewald. 1999. The MHC of a broiler chicken line: serology, B-G Genotypes, and B-F/B-LB sequences. Available: **http://www.vetmed.auburn.edu/~ewaldsj/html**, March 15,2002.

Livant, E.J., D. Zheng, L.W. Johnson, W. Shi, and S.J. Ewald. 2001. Three new MHC haplotypes in broiler breeder chickens. **Anim. Gen**. 32:123-131.

Lipkind, M., H. Burger, R. Rott, and C. Scholtissek. 1984. Genetic characterization of influenza A viruses isolated from birds in Israel. A contribution to the ecology of avian influenza viruses. **Zentralblatt fur Veterinarmedizin, B.** 31: 721-728. Abstr.

Lyon, J.A. and V.S. Hinshaw. 1991. Replication of influenza A viruses in an avian macrophage cell line**. J. Gen. Virol**. 72: 2011-2013.

Martin, A., W.B. Gross, E.A. Dunnington, R.W. Briles, W.E. Briles, and P.B. Siegel. 1986. Resistant to natural and controlled exposures to *Eimeria tenella*: Genetic variation and alloantigenic systems. **Poult. Sci.** 65: 1847-1852.

McConnell, S.K., D.A. Dawson, A. Wardle, and T. Burke. 1999. The isolation and mapping of 19 tetranucleotide microsatellite chicken. **Anim. Gen.** 30: 183-189.

Miggiano, V.C., J. Bergen, and J.R.L. Pink. 1974. The mixed leukocyte reaction in chickens. Evidence for control by the major histocompatability complex. **Eur. J. immunol.** 4: 397-401.

Miller, M.M., H. Abplanalp, and R. Goto. 1988. Genotyping chickens for the B-G subregion of the major histocompatibility complex using restriction fragment length polymorphisms. **Immunogenetics** 28: 374-379.

______, L.D. Bacon, K. Hala, H.D. Hunt, S.J. Ewald, J. Kaufman, R. Zoorob, and W.E. Briles. 2004. Nomenclature for the chicken major histocompatibility (B and Y) complex. **Immunogenetics.** 56: 261-279.

______, R. Goto, A. Bernot, R. Zoorob, C. Auffray, N. Bumstead, and W.E. Briles. 1994a. Two Mhc class I and two Mhc class II genes map to the chicken Rfp-Y system outside the B complex. **Proc. Natl. Acad. Sci.** 91: 4397-4401.

_______, R. Zoorob, C. Auffray and W.E. Briles. 1994b. Regions of homology shared by Rfp-Y and major histocompatibility B complex genes. **Immunogenetics** 39: 71-73.

_______, R.L.Jr. Taylor, R. Zoorob, C. Auffray, R.W. Briles, W.E. Briles, and S. Bloom. 1996. Assignment of Rfp-Y to the chicken major histocompatibility complex/NOR microchromosome and evidence for high-frequency of recombination associated with the nucleolar organizer region. **Proc. Natl. Acad. Sci.** 93: 3958-3962.

Nei, M. 1972. Genetic Distance between populations. **American Naturalist** 106: 283-292.

Nishibori, M., S. Nakaki, M. Tsudzuki, and Y. Yamamoto. 2000. Utility of three restriction fragment length polymorphism probes for genotyping of the chicken major histocompatibility complex class IV region. **Poult. Sci**. 79: 305-311.

Nordskog, A.W., I.Y. Pelzer, and S.J. Lamont. 1987. Subregions and functions of the chicken major histocompatibility complex. **Poult. Sci**. 66: 790-794.

Pazderka, F., B.M. Longenecker, G.R.J. Law, H.A. Stone, and R.F. Ruth. 1975. Histocompatibility of chicken populations selected for resistance to Marek's disease. **Available: http://www.nature.com/cgi-taf/DynaPage.taf?file=/nature/journal/v401/n6756/full/401923a0_r.html**, March 15,2002.

Pharr, G.T., A.V. Gwynn, and L.D. Bacon. 1996. Histocompatibility antigen(s) linked to Rfp-Y (Mhc-like) genes in the chicken. **Available: http://www.nal.usda.gov/ttic/tektran/data/000006/69/0000066925.html**, March 15,2002.

_______, H.D. Hunt, L.D. Bacon, and J.B. Dodgson. 1993. Identification of class II major histocompatibility complex polymorphisms predicted to be important in peptide antigen presentation. **Poult. Sci**. 72: 1312-1317.

_______, J.B. Dodgson, H.H. Hunt, and L.D. Bacon. 1998. Class II MHC cDNAs in 15I5 B-congenic chickens. **Immunogenetics.** 47:350–354.

_______, L.D. Bacon, and J.B. Dodgson. 1994. A classI cDNA from SPAFAS line-11 chickens. **Available: http://genomics.cas.psu.edu/vitae.html**, March 15,2002.

Pink, J.R.L., W. Droege, K. Hala, V.C. Miggiano, and A. Ziegler. 1977. A three locus model for the chicken major histocompatibility complex. Available: **http://www.nature.com/cgi-taf/DynaPage.taf?file=/nature/journal/v401/n6756/full/401923a0_r.html**, March 15,2002.

Powell, P.C. 1984. Marek's disease and its causative virus Pages 155-183 **in: Leukemia and Lymphoma Research. Vol.1. Mechanisms of Viral Leukaemogensis.** J.M.Goldman and O.Jarrett, ed. Churchill Livingstone, Edinburgh, Scotland, and New York, NY.

Quddus, J., S. Prakash, R. Bahn, S. Banerjee, and C. David. 1986. Genetics of immune response and Susceptibility to disease. Vol.XI. Pages 593-613 **in: 3rd World Congress on Genetics Applied to Livestock Production.** G.E. Dickerson and R.K. Johnson, ed. Univ. Nebraska, Lincoln, NE.

Rothschild, M.F. 1989. Selection for disease resistance in the pig. Available: http://mark.asci.ncsu.edu/nsif/98proc/rothschild.htm, March 15,2002.

Roslin Bioinformatics Group. 1999. Chicken genome. Available: http://www.ri.bbsrc.ac.uk/chickmap/html, March 15,2002.

Sambrook, J., E. F. Fritsch, and T. Manlatis. 1989. **Molecular cloning: a laboratory manual. 2nd ed.** Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Simonsen, M., K. Hala, and E.M. Nicolaisen. 1980. Linkage disequilibrium of MHC genes in the chicken. I. The B-F and B-G loci. **Available: http://www.poultryscience.org/psa/toc/papers/98/ps981858.pdf.html**, March 15,2002.

______, M. Crone, C. Koch, and K. Hala. 1982. The MHC haplotypes of the chicken. **Available: http://www.poultryscience.org/psa/toc/papers/98/ps981858.pdf.html**, March 15,2002.

Spike, C.A. and S.J. Lamont. 1995. Genetic analysis of three loci homologous to human G9a: evidence for linkage of a class III gene with the chicken MHC. **Anim. Gen.** 26:185-187.

Sung, A.M., A.W. Nordskog, S.J. Lamont, and C.M. Warner. 1993. Isolation and charaterization of cDNA Clones for chicken major histocompatibility complex classII molecules. **Anim. Gen.** 24: 227-233.

Swayne, D.E., D.L. Suarez, E. Hoffmann, R.G. Webster. 2003. Land-based birds as potential disseminators of avian/mammalian reassortant influenza A viruses. **Avian Dis**. 47: 1114-1117.

Uni, Z., M. Gutman, G. Leitner, E. Landesman, D. Heller, and A. Cahaner. 1993. Major histocompatibility complex class IV restriction fragment length polymorphism markers in replicated meat-type chicken lines divergently selected for high or low early immune response. **Poult. sci.** 72: 1823-1831.

van der Zijpp, A.J. 1983. Breeding for immune responsiveness and disease resistance. **Available: http://www.bsas.org.uk/publs/genchng/paper8.pdf.html,** March 15,2002.

Wain, H.M., 1997. Targeted mapping of the chicken genome. **Ph.D Thesis. The University of Hertfordshire Hatfield, Hertfordshire**. 90p.

Warner, C., B. Gerndt, Y. Xu, Y. Bourlet, C. Auffray, S. Lamont, and A. Nordskog. 1989. Restriction fragment length polymorphism analysis of major histocompatibility complex class II genes from inbred chicken lines. **Available: http://www.poultryscience.org/psa/toc/papers/97/ps971517.pdf.html,** March 15,2002.

Xu, Y., J. Pitcovski, L. Peterson, C. Auffray, Y. Bourlet, B.M. Gerndt, A.W. Nordskog, S.J. Lamont, and C.M. Warner. 1989. Isolation and characterization of three class II MHC genomic clones from the chicken. **J. Immunol**. 142: 2122-2132.

Yonash, N., E.D. Heller, J. Hillel, and A. Cahaner. 2000. Detection of RFLP markers associated with antibody response in meat-type chickens: haplotype/genotype, single-band, and multiband analyses of RFLP in the major histocompatibility complex. **J. Hered.** 91:24-30.

Zheng, D., G. O'Keefe, L.Li, L.W. Johnson, and S.J. Ewald. 1999. A PCR method for typing B-L beta II family (class II MHC) alleles in broiler chickens. **Anim Genet.** 30:109-119.

Zoorob, R., A. Bernot, D.M. Renoir, F. Choukri, and C. Auffray. 1993. Chicken major histocompatibility Complex classII B genes: analysis of interallelic and inter-locus sequence variance. **Euro. J. Immunol.** 23: 1139-1145.

______, G. Behar, G. Kroemer, and C. Auffray. 1990. Organization of a functional chicken classII B gene. . Available: **http://eebweb.arizona.edu/nachman/hoekstra/PDFs/MHC.pdf.html,** March 15,2002.

APPENDIX

APPENDIX 1

Appendix Table 1 Nucleotides and amino sequencing of MHC class I haplotypes (BFα1 exon2)

Haplotype	Accession no.[1/]	Nucleotide	Amino acid	Function [2/]
A1		**GTGGACGGGGAACTCTTC**A TGCACTACAACAGGACCGCG CGGAGGGATGTGCCCCGCAC CGAGTGGATGGCGGCCAACG CGGACCAGCAGTACTGGGAC AGAGAGACGCAGATCGCACC AGGGAAAGGAGAGATTGACC GCGAGACCGGGGGTACAT**TG** **CAGCGGCGCTACAACCAGA**	V D G E L F **Met** H Y N R T A R R D V P R T E W **Met** A A N A D Q Q Y W D R E T Q I A P G K G E I D R E T G G T L Q R R Y N Q	PBS
A9	DQ011916	**GTGGACGGGGAACTCTTC**A TGCATTACAACAGCACCGCG CGGAGGGCTGTGCCCCGCAC CGAGTGGATGGCGGCCAAGG CGGACCAGCAGTACTGGGAT GGACAGACGCAGCTGGGCCA GGGCCATGAGCAGGTTAACA GCGAGGACCTGGACACAC**TG** **CAGCGGCGCTACAACCAGA**	V D G E L F **Met** H Y N S T A R R A V P R T E W **Met** A A K A D Q Q Y W D G Q T Q L G Q G H E Q V N S E D L D T L Q R R Y N Q	PBS
B12	DQ011917	**GTGGACGGGGAACTCTTC**GT GCACTACAACAGCACCGCGCG GAGGTACGTGCCCCGCACCGA GTGGATAGCGGCCAAGGCGGA CCAGCAGTACTGGCATGGACA GACGCATATCGGACAGGGCAA TGAGCAGATTGACCCGCGAGA ACCTGGGCATA**TGCAGCGGC** **GCTACAACCAGA**	V D G E L F V H Y N S T A R R Y V P R T E W I A A K A D Q Q Y W H G Q T H I G Q G N E Q I D P R E P G H **Met** Q R R Y N Q	PBS
B13	DQ011918	**GTGGACGGGGAACTCTTC** GTGCACTACAACAGCACCG CGCGGAGGTACGTGCCCCG CACCGAGTGGATAGCGGCC NACACGACCCAGCAGTACT GGGATGGACAGACGCAGAT CGGACAGCTCAATGAGCAG ATTAACCGCGAGAACCTGG GCATAC**GGCAGCGGCGCT** **ACAACCAGA**	V D G E L F V H Y N S T A R R Y V P R T E W I A A X T T Q Q Y W D G Q T Q I G Q L N E Q I N R E N L G I R Q R R Y N Q	PBS

Appendix Table 1 (Continued)

Haplotype	Accession no.[1/]	Nucleotide	Amino acid	Function [2/]
B14		**GTGGACGGGGAACTCTTC**GT GCACTACAACAGCACCGCGCG GAGGTTTGTGCCCCGCACCGA GTGGATGGCGGCCAAGGCGG ACCAGCAGTACTGGCACAGA **N**TGACGCANATCGGACAGCG CACTGAGCAGATTGACCGCG ATGACCTGGGCACAC**TGCAG** **CGGCGCTACAACCAGA**	V D G E L F V H Y N S T A R R F V P R T E W **Met** A A K A D Q Q Y W H R X T X I G Q R T E Q I D R D D L G T L Q R R Y N Q	PBS
B17	DQ011919	**GTGGACGGGGAACTCTTC**A TGCATTACAACAGCATCCGC GCGGAGGTCTGTGCCCCGCA CCGAGTGGATGGCGGCCAAT GGCGGACCAGCAGTACCTGG GATGGACAGACGCAGATCGA ACACAATGAGCAGATTGACC GCCCGCGAGACCTGGGAT**TG** **CAGCGGCGCTACAACCAGA**	V D G E L F **Met** H Y N S I R A E V C A P H R V D G G Q W R T S S T W D G Q T Q I E H N E Q I D R P R D L G L Q R R Y N Q	PBS
B18		**GTGGACGGGGAACTCTTC**G TGCACTACAACAGCACCGC GCGGAGGGCTGTGCCCCGC ACCGAGTGGATGGCGGCCA AGGCGGACCAGCAGTACTG GGATGGACAGACGCAGATC GTACAGGGCCGTGAGCAGG TTATCCGCGAGAACCTGGA CATAC**TGCAGCGGCGCTA** **CAACCAGA**	V D G E L F V H Y N S T A R R A V P R T E W **Met** A A K A D Q Q Y W D G Q T Q I V Q G R E Q V I R E N L D I L Q R R Y N Q	PBS

Appendix Table 1 (Continued)

Haplotype	Accession no.[1/]	Nucleotide	Amino acid	Function [2/]
B19		**GTGGACGGGGAACTCTTC**GT GCACTACAACAGCACCGCGCG GAGGTACGTGCCCCGCACCGA GTGGATAGCGGCCAAGGCGGA CCAGCAGTACTGGTACAGACA GACGCAGATCGCACAGAGCAG TGAGCATGATGACCGCGACAA CCTGGGCATAC**TGCAGCGGC GCTACAACCAGA**	V D G E L F V H Y N S T A R R Y V P R T E W I A A K A D Q Q Y W Y R Q T Q I A Q S S E H D D R D N L G I L Q R R Y N Q	PBS
B21	DQ011921	**GTGGACGGGGAACTCTTC** ATGCACTACAACAGCACCG CTCGGAGGGATGTGCCCCG CACCGAGTGGATGGCGGCC ACACGACACCAGCAGTACT GGGACAGAGAGACGCAGAT CGTACAGGGCAGTGAGCAG ATTAACCGCGAGAACCTGGA CATAC**TGCAGCGGCGCTAC AACCAGA**	V D G E L F **Met** H Y N S T A R R D V P R T E W **Met** A A T R H Q Q Y W D R E T Q I V Q G S E Q I N R E N L D I L Q R R Y N Q	PBS
B6		**GTGGACGGGGAACTCTTC**G TGCACTACAACAGCACCGCG CGGAGGGTTGTGCCCCGCAC CCGAGTGGATGGCGGCCAAT GGCGGACCAGCAGTACTGGG ACAGAGAGACCGCAGGTCGC AGGCAATGAGCAGATTGACC GCGATCGATACCTGGCAC**TG CAGCGGCGCTACAACCAGA**	V D G E L F V H Y N S T A R R V V P R T R V D G G Q W R T S S T G T E R P Q V A G N E Q I D R D R Y L A L Q R R Y N Q	PBS

Appendix Table 1 (Continued)

Haplotype	Accession no.[1/]	Nucleotide	Amino acid	Function [2/]
BA4v	DQ011925	**GTGGACGGGGAACTCTTC**AC GCACTACAACAGCACCGCGCG GAGGGACACGCCCCGCACCGA GTGGATAGCGGCCAGGCGGAA CCAGCAGTACTGGGACAGACA GACGCAGACCTCACAGCGCAC TGAGCAGATTGACCGCGATGA CCTGGGCACAC**TGCAGCGGC GCTACAACCAGA**	V D G E L F T H Y N S T A R R D T P R T E W I A A R R N Q Q Y W D R Q T Q T S Q R T E Q I D R D D L G T L Q R R Y N Q	PBS
BA12	DQ011924	**GTGGACGGGGAACTCTTC** GTGCACTACAACAGCACCG CGCGGAGGGTTGTGCCCCG CACCGAGTGGATGGCGGCC AACACGGACCAGCAGTACT GGGAACAGATAGACCAGAT CGTACAGGGCCGTGAGCAG ATTGACCGCGAGAACCTGG GCATAC**TGCAGCGGCGCT ACAACCAGA**	V D G E L F V H Y N S T A R R V V P R T E W **Met** A A N T D Q Q Y W E Q I D Q I V Q G R E Q I D R E N L G I L Q R R Y N Q	PBS
BC1	DQ011926	**GTGGACGGGGAACTCTTC**AT GCACTACAACAGCACCGCNCG GAGGGACGTGCCCCGCACCGA GTGGATGGCAGCCAACACGGA CCAGCAGTACTGGGACAGAGT GACGCAGATCGGACAGGGCCA TGAGCAGACTGACCACGGGAA CCTGGACACAC**TGCAGCGGCG CTACAACCAGA**	V D G E L F **Met** H Y N S T X R R D V P R T E W **Met** A A N T D Q Q Y W D R V T Q I G Q G H E Q T D H G N L D T L Q R R Y N Q	PBS

Appendix Table 1 (Continued)

Haplotype	Accession no.[1/]	Nucleotide	Amino acid	Function [2/]
BC2v		**GTGGACGGGGAACTCTTC** GTGCACTACAACAGCACCG CGCGGAGGTACGTGCCCCG CACCGAGTGGATGGCAGCC AGGGCGGACCGGCAGTACT GGGATGGACAGACGCATAT CGGACAGCGCAATGAGCAG GTTATCCGCGAGAGCCTGG GCACAC**TGCAGCGGCGCT ACAACCAGA**	V D G E L F V H Y N S T A R R Y V P R T E W **Met** A A R A D R Q Y W D G Q T H I G Q R N E Q V I R E S L G T L Q R R Y N Q	PBS
BC7	DQ011927	**GTGGACGGGGAACTCTTC**A TGCACTACAACAGCACCGCG CGGAGGTACGTGCCCCGCAC CGAGTGGATGGCGGCCAACA CGGACCAGCAGTACTGGGAT GGACAGACGCAGATCGGACA GGGCCATGAGCAGGTTGACC GCGAGAACCTGGGCACACTC CA**GCGGCGCTACAACCAGA**	V D G E L F **Met** H Y N S T A R R Y V P R T E W **Met** A A N T D Q Q Y W D G Q T Q I G Q G H E Q V D R E N L G T L Q R R Y N Q	PBS

[1/] Accession no. were conceded by Genbank
[2/] PBS = Pathogen binding site

Appendix Table 2 Nucleotides and amino sequencing of MHC class II haplotypes (BLβ1 exon2)

Haplotype	Accession no.[1/]	Nucleotide	Amino acid	Function [2/]
B12	AY993991	**GTGCCCGCAGCGTTCTTC** TTCTGCGGTCGGATATCCG AGTGCCACTACCTGAACGG CACCGAGCGGGTGAGGTTT CTGGACAGGTACATCTACA ACCGGCAGCAGTTCACGCA CTTCGACAGCGACGTGGGG AAATTTGTGGCCGATACAC CGCTGGGTGAGCCGCAAGC TGAATACTGGAACAGCAAC GCCGAGTTTCTGGAGAACC GAATGAATGAAGTGGACAG GTTCTGCCGGCACAACTACG GGGTTGTGGAGT**CCTTCAC** **GGTGCAGAGGA**	V P A A F F F C G R I S E C H Y L N G T E R V R F L D R Y I Y N R Q Q F T H F D S D V G K F V A D T P L G E P Q A E Y W N S N A E F L E N R **Met** N E V D R F C R H N Y G V V E S F T V Q R	PBS
B19	AY993992	**GTGCCCGCAGCGTTCTTC** TATACGGGCGTGATATATG AGTGCCACTACCTGAACGG CACCGAGCGGGTGAGGTTT CTGGACAGGGAAATCTACA ACCGGCAGCAGTTCGTGCA CTTCGACAGCGACGTGGGG AAATACGTGGCTGATACAC CGCTGGGTGAGCCTCAGGT TGAATTCTGGAACAGCGAC GCCGAGTTTCTGGAGAACC AAATGAATGAAGTGGACAG GTTCTGCCGGCACAACTAC GGGGTTGTGGAGT**CCTTCA** **CGGTGCAGAGGA**	V P A A F F Y T G V I Y E C H Y L N G T E R V R F L D R E I Y N R Q Q F V H F D S D V G K Y V A D T P L G E P Q V E F W N S D A E F L E N Q **Met** N E V D R F C R H N Y G V V E S F T V Q R	PBS

Appendix Table 2 (Continued)

Haplotype	Accession no.[1/]	Nucleotide	Amino acid	Function [2/]
B21	AY993993	**GTGCCCGCAGCGTTCTTC** CAGTGGACTTTTAAAGCAG AGTGCCACTACCCGAACGG CACCGAGCGGGTGAGGTAT CTGGTCAGGTATGTCTACAA CCGGCAGGAGTACGCGCAC TTCGACAGCGACGTGGGGA AACACGTGGCTGATACACC GCTGGGTGAGCCTCAAGCTG AATACTGGAACAGCAACGCC GAGATTCTGGAGAACCGAAT GAATGAAGTGGACACGTTCT GCCGGCACAACTACGGGG TTGTGGAGT**CCTTCACGG** **TGCAGAGGA**	V P A A F F Q W T F K A E C H Y P N G T E R V R Y L V R Y V Y N R Q E Y A H F D S D V G K H V A D T P L G E P Q A E Y W N S N A E I L E N R **Met** N E V D T F C R H N Y G V V E S F T V Q R	PBS
B14	AY993997	**GTGCCCGCAGCGTTCTTC**T TCTACGGTATGATATTCGAG TGCCACTACCTGAACGGCAC CGAGCGGGTGAGGTATCTGG ACAGGTATATCTACAACCGG CAGGAGTTCACGCACTTCGA CAGCGACGTGGGGAAATACG TGGCTGATACACCGCTGGGT GAGCCTCAAGCTGAATACTG GAACAGCAACGCCGAGTTTC TGGAGAACCGAATGAATGAA GTGGACACGTTCTGCCGGCAC AACTACGGGGTTGGGGAGT**C** **CTTCACGGTGCAGAGGA**	V P A A F F F Y G **Met** I F E C H Y L N G T E R V R Y L D R Y I Y N R Q E F T H F D S D V G K Y V A D T P L G E P Q A E Y W N S N A E F L E N R **Met** N E V D T F C R H N Y G V G E S F T V Q R	PBS

Appendix Table 2 (Continued)

Haplotype	Accession no.[1/]	Nucleotide	Amino acid	Function [2/]
A9	AY973596	**CGTTCTTCTTCTACGGTAAG** **AT**AGGTGAGTGCCACTACCT GAACGGCACCGAGCGGGTGA GGTCCGCACAGGGACAAGCT ACAATTCGGCAGCAGTACGC GCACTTCGACAGCGACGTGG GGAAATTCGTGGCCGATACA CCGCTGGGTGAGCGTCAGGC TGAATACTGGAACAGCAACG CCGAGATTCTGGAGAACCAA ATGAATGAAGTGGA**CAGGG** **TCTGCCGGCACAACTA**	F F F Y G K I G E C H Y L N G T E R V R S A Q G Q A T I R Q Q Y A H F D S D V G K F V A D T P L G E R Q A E Y W N S N A E I L E N Q **Met** N E V D R V C R H N	PBS
BA12	AY973602	**CGTTCTTCTTCTGCGGT** **GCGAT**ATTTGAGTGCCAC TACCTGAACGGCACCGAG CGGGTGAGGTTTCTAACC GGGCAAATTTACAACCGG CAGCAGTACGCGCACTTC GACAGCGACGTGGGGAAA TACGTGGCTGATACACCG CTGGGTGAGCGTCAGGCTG AATACTGGAACAGCAACGC CGAGTTTCTGGAGAACCGA ATGAATGAAGTGGAC**ACGT** **TCTGCCGGCACAACTA**	F F F C G A I F E C H Y L N G T E R V R F L T G Q I Y N R Q Q Y A H F D S D V G K Y V A D T P L G E R Q A E Y W N S N A E F L E N R **Met** N E V D T F C R H N	PBS

[1/] Accession no. were conceded by Genbank
[2/] PBS = Pathogen binding site

Appendix Table 3 MHC class I haplotypes in LHK chicken of DLD

MHC haplotypes	No. of chicken	Percent in herd
A9A9	6	7.89
A9B12	1	1.32
A9B14	2	2.63
A9B18	1	1.32
A9BA12	1	1.32
A9BC1	6	7.89
B12B12	3	3.95
B12B5	1	1.32
B12B6	1	1.32
B13B13	7	9.21
B13BC1	2	2.63
B14B18	3	3.95
B14BC1	2	2.63
B17B17	1	1.32
B19BA4v	1	1.32
B21B21	10	13.16
B21BA12	4	5.26
B21BC1	1	1.32
B2B2	3	3.95
B5B5	1	1.32
B5BA12	1	1.32
BA12BC1	2	2.63
BA1BA1	1	1.32
BA4vBA4v	2	2.63
BC1BC1	11	14.47
BC7BC7	2	2.63

Appendix Table 4 MHC class II haplotypes in LHK chicken of DLD

MHC haplotypes	No. of chicken	Percent in herd
A9A9	3	3.95
B12B12	1	1.32
B12B2	10	13.16
B12B21	3	3.95
B14B14	2	2.63
B14B2	2	2.63
B14B21	3	3.95
B14B5	1	1.32
B14BA12	2	2.63
B19B19	6	7.89
B21B21	19	25.00
B21B5	3	3.95
B21BA12	1	1.32
B2B2	1	1.32
B2B21	10	13.16
B2B5	5	6.58
B5B5	1	1.32
BA12BA12	3	3.95

Appendix Table 5 MHC class I haplotypes in PHD chicken of DLD

MHC haplotypes	No. of chicken	Percent in herd
A9A9	4	5.00
A9B12	3	3.75
A9B14	2	2.50
A9B18	1	1.25
A9B5	2	2.50
A9BA12	2	2.50
A9BC1	3	3.75
B12B12	6	7.50
B12B21	2	2.50
B12B5	1	1.25
B12B6	1	1.25
B13B13	4	5.00
B13BC1	3	3.75
B13BC2v	1	1.25
B14B18	11	13.75
B14BC1	1	1.25
B18BA12	1	1.25
B21B21	14	17.50
B21BA12	4	5.00
B21BC7	2	2.50
B2B2	1	1.25
B5B5	1	1.25
B5BA12	3	3.75
BC1BA12	1	1.25
BC1BC1	6	7.50

Appendix Table 6 MHC class II haplotypes in PHD chicken of DLD

MHC haplotypes	No. of chicken	Percent in herd
A9A9	1	1.25
B12B12	3	3.75
B12B21	2	2.5
B12B21	3	3.75
B14B14	3	3.75
B14B2	4	5
B14B21	5	6.25
B14B5	2	2.5
B19B19	2	2.5
B19B2	3	3.75
B21B21	15	18.75
B21B5	5	6.25
B21BA12	2	2.5
B2B2	6	7.5
B2B21	7	8.75
B2B5	12	15
B5B5	2	2.5
BA12BA12	3	3.75

Appendix Table 7 MHC class I haplotypes in KL chicken of DLD

MHC haplotypes	No. of chicken	Percent in herd
A9A9	4	10.53
A9B18	1	2.63
A9B5	1	2.63
A9BA12	1	2.63
A9BC1	1	2.63
B12B12	1	2.63
B13B13	2	5.26
B17BA12	4	10.53
B19BC1	1	2.63
B21B21	6	15.79
B21BA12	1	2.63
B21BC7	1	2.63
B2B2	2	5.26
B5B5	3	7.89
B5BA12	2	5.26
BA12BC1	2	5.26
BA1BA1	1	2.63
BA4vBA4v	1	2.63
BC1BC1	3	7.89

Appendix Table 8 MHC class II haplotypes in KL chicken of DLD

MHC haplotypes	No. of chicken	Percent in herd
B12B12	2	5.26
B12B19	1	2.63
B12B2	1	2.63
B12B5	1	2.63
B14B21	1	2.63
B14B5	1	2.63
B19B19	2	5.26
B19B5	1	2.63
B21B21	14	36.84
B21B5	7	18.42
B2B21	5	13.16
B2B5	1	2.63
BA12BA12	1	2.63

Appendix Table 9 MHC class I haplotypes in ND chicken of DLD

MHC haplotypes	No. of chicken	Percent in herd
A9A9	1	3.57
A9B14	2	7.14
A9B18	1	3.57
A9B5	3	10.71
A9BC1	1	3.57
B13B21	2	7.14
B13BC1	2	7.14
B13BC2v	2	7.14
B21B21	3	10.71
B21BC1	3	10.71
B5BA12	2	7.14
BC1BC1	6	21.43

Appendix Table 10 MHC class II haplotypes in ND chicken of DLD

MHC haplotypes	No. of chicken	Percent in herd
B12B12	6	21.43
B12B14	1	3.57
B12B19	2	7.14
B12B2	3	10.71
B12B21	3	10.71
B12B5	1	3.57
B14B14	1	3.57
B14B5	3	10.71
B19B19	2	7.14
B19B5	2	7.14
B21B21	1	3.57
B21B4	1	3.57
B21B5	1	3.57
B4B5	1	3.57

Appendix Table 11 MHC class I haplotypes in CH chicken of DLD

MHC haplotypes	No. of chicken	Percent in herd
A9A9	1	3.33
A9B21	2	6.67
A9B5	2	6.67
A9BC1	6	20.00
B13B13	1	3.33
B13B21	1	3.33
B13BC1	3	10.00
B14BC1	2	6.67
B21BC1	1	3.33
BC1BC1	10	33.33
B5BA12	1	3.33

Appendix Table 12 MHC class II haplotypes in CH chicken of DLD

MHC haplotypes	No. of chicken	Percent in herd
B12B12	1	0.30
B12B14	2	0.60
B12B2	2	0.60
B12B5	7	2.10
B14B2	2	0.60
B14B21	2	0.60
B14B5	3	0.90
B14BA12	1	0.30
B19B19	1	0.30
B19B5	2	0.60
B21B21	3	0.90
B2B21	1	0.30
B2B5	1	0.30
B5B5	2	0.60

APPENDIX 2

Appendix Figure 1 Nucleotide sequencing of MHC class II exon2 270 bp plus with 10 nucleotide of intron in region of forward primer

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