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Advances in methodologies for detecting MHC-*B* variability in chickens

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ABSTRACT The chicken major histocompatibility B complex (**MHC-*B***) region is of great interest owing to its very strong association with resistance to many diseases. Variation in the MHC-*B* was initially identified by hemagglutination of red blood cells with specific alloantisera. New technologies, developed to identify variation in biological materials, have been applied to the chicken MHC. Protein variation encoded by the MHC genes was examined by immunoprecipitation and 2-dimensional gel electrophoresis. Increased availability of DNA probes, PCR, and sequencing resulted in the application of DNA-based methods for MHC detection. The chicken reference genome, completed in 2004, allowed further refinements in DNA methods that enabled more rapid examination of MHC variation and extended such analyses to include very diverse chicken populations. This review progresses from the inception of MHC-*B* identification to the present, describing multiple methods, plus their advantages and disadvantages.

Key words: chicken MHC-*B*, haplotypes, detection methods, review

INTRODUCTION

The chicken major histocompatibility B complex (**MHC-*B***) is a cluster of genes, many of which are polymorphic and are involved with immune response. These genes encode proteins that are essential for the immune system to recognize antigens expressed by cells and determine whether these are foreign proteins (from invading pathogens) and should be destroyed. The chicken MHC-*B* was first identified as one of the several erythrocyte antigens or blood group systems by Briles et al., (1950). Later studies showed that skin graft rejection was linked to B system differences and thus the B blood group system was identified as the chicken MHC-*B* (Schierman and Nordskog, 1961). The serendipitous observation of recombinants within the MHC-*B* indicated that the erythrocyte antigen specificity and tissue histocompatibility was determined by separate but closely linked chromosomal regions. The erythrocyte antigen specificity region was defined as BG, the histocompatibility region was defined as BL (equivalent to mammalian class I) with expression detected on both erythrocytes and lymphocytes and a third region, BL (equivalent to mammalian class II) was identified with antigenic expression limited to lymphocytes and monocytes/macrophages (Briles and Briles, 1987).

The chicken MHC is now known to consist of 2 regions, MHC-*B* and MHC-*Y*, both found on chromosome 16, but with independent assortment (Briles et al., 1993; Miller et al., 1996). Less information is known for the MHC-*Y* region genes, but they are polymorphic, are structurally similar to many MHC-*B* genes, and can be alloantigenic. The status of knowledge of the MHC-*B* and MHC-*Y* was recently summarized by Miller and Taylor (2016).

The sequence of the MHC-*B* region was reported by Shiina et al. (2007) and contains 46 genes within a 242,000 base region. Comparison of sequences for this MHC-*B* region from multiple haplotypes reveals gene structural diversity, insertions and deletions, as well as gene rearrangements between different haplotypes (Hosomichi et al., 2008). The considerable allelic diversity within coding regions is likely due to subsequent selection for variation in response to pathogen challenge.

The chicken MHC-*B* has been of particular interest because of its strong associations with resistance to multiple pathogens that can impact poultry production. Hansen et al. (1967) first reported an association between MHC-*B* variation and disease resistance in elite lines at Hy-Line International following challenge with Marek's disease virus (**MDV**). Multiple studies since

have confirmed this relationship between MHC-*B* haplotypes and MDV resistance (Briles et al., 1977; Bacon et al., 1981; 1983). Furthermore, MHC-*B* influence on efficacy of Marek's disease vaccine has also been demonstrated (Bacon and Witter 1993; 1994). Resistance to numerous other viral diseases has also been shown to be associated with MHC-*B* variation including avian leukosis virus (Bacon et al., 1981; Yoo and Sheldon, 1992), Newcastle disease virus (Dunnington et al., 1992; Lwelamira et al., 2008; Norup et al., 2011), infectious bursal disease virus (Juul-Madsen et al., 2002), fowl pox (Lee et al., 2004), and infectious bronchitis virus (Bacon et al., 2004). MHC-*B* variation also influences Rous sarcoma virus–induced tumor growth as well as metastasis (Collins et al., 1977, 1979; Schierman et al., 1977; Bacon et al., 1981; Taylor et al., 1992; Taylor, 2004; Suzuki et al., 2010). Disease resistance associated with MHC-*B* haplotype has been reported for numerous bacteria-induced diseases, including *Staphylococcus aureus* infections (Cotter et al., 1992), *Salmonella* spp. (Cotter et al., 1998; Lamont et al., 2002; Zhou and Lamont 2003; Schou et al., 2010), and *Pasteurella multocida* (Lamont et al., 1987; Schou et al., 2010). Associations with severity of necrotic enteritis induced by *Clostridium perfringens* as well as *Escherichia coli*–induced cellulitis and mortality have also been reported (Siegel et al., 1993; Macklin et al., 2002, 2009; Cavero et al., 2009). Associations between MHC-*B* haplotype and the intestinal worm *Ascaridia galli* have been reported in indigenous and exotic chickens in Vietnam (Schou et al., 2007; 2010). MHC-*B* haplotype influences on *Eimeria* infections were identified for cecal lesions or fecal parasite numbers after experimental infection (Clare et al., 1985; Lillehoj et al., 1988; Caron et al., 1997; Kim et al., 2008). MHC-*B* influences on Northern fowl mite infestations in both MHC-*B* congenic lines and commercial out-crossed chickens have also been found (Owen et al., 2008).

Variation within the chicken MHC-*B* was originally detected with alloantisera produced after immunizations of blood between birds with different MHC-*B* haplotypes (Briles et al., 1950; Gilmour, 1959). MHC-*B* detection methods have evolved over the past 60 yr. This review focuses on the methods used to detect chicken MHC-*B* variation, from the early alloantisera detection of protein variants to the current methods of DNA-based detection of SNP within the MHC-*B* region. With the recent decline in antibiotic and antiparasitic drugs allowed for treatment of poultry diseases, and the demonstrated relationship between MHC-*B* variation and disease resistance in poultry, the MHC-*B* is even more relevant to the poultry breeding and production industries.

PROTEIN VARIATION

Serological Detection

The chicken MHC-*B* was originally identified as one of the several blood group loci that were defined by the use of specific alloantisera. This alloantiserum was produced by a recipient bird after multiple blood cell immunizations from a donor. The recipient bird sera would cause agglutination when mixed with blood cells from the donor, but not those of the recipient, indicating antigenic differences between donor and recipient. Multiple blood groups were identified using these alloantisera. Repeated adsorptions of the sera with blood cells from other related individuals were often necessary to remove antibodies that reacted to other non-B blood group antigenic differences and ensure the specificity of the reagent to the B blood group system (Briles et al., 1950).

Multiple laboratories were independently investigating blood group variation in different chicken stocks. In 1982, comparisons of numerous reagents from multiple sources resulted in the serological identification of 27 B reference haplotypes, 78% of which were found within the White Leghorn breed (Briles and Briles, 1982). This required the exchange of fresh blood cells, and specific antiserum across international boundaries. The large body of information primarily from one breed was probably due to a variety of factors including limited lines available at the multiple laboratories studying immunological variation in chickens, commercial use of the White Leghorn breed for white egg production, and the access to elite White Leghorn lines provided by 2 commercial layer breeding companies (Hy-Line and DeKalb). The remaining unique MHC-*B* haplotypes were identified within the New Hampshire breed (6) and Ancona breed (2). Unfortunately, many of the stocks originally identified as sources of these standard haplotypes are no longer in existence and thus the haplotypes are unavailable for study.

The production of MHC-*B*-specific alloantiserum and interpretation of the agglutination results is as much art as it is science. Frequently, different haplotypes could show agglutination by the same specific antiserum (cross reactions). Judicious adsorptions of these antisera could remove the cross-reactive antibodies (Briles and Briles 1982). Some of the cross reactions can be due to antigenic similarities between proteins that differed between different MHC-*B* haplotypes (Fulton et al., 1995; 2001). The variability inherent in the production of biological reagents can often make it difficult to produce more antiserum with the identical specificity. Even the use of MHC-*B* congenic lines to produce antisera does not ensure consistent MHC-*B* alloantisera production (Fulton et al., 1996).

Chickens also contain 12 non-MHC blood groups, many of which are highly polymorphic (see review by Taylor et al., 2016). These blood groups are also highly antigenic, and if they are not matched between donor and recipient, the subsequent antisera produced can contain antibodies specific for these non-MHC blood groups. The use of inbred MHC-*B* congenic lines which would be identical for all non-MHC antigens will eliminate this problem. However, the presence of these non-B blood groups in outbred lines is a serious impediment to the production of MHC-specific antisera in multiple

lines, adding complexity to production of antisera, and interpretation of results. This is another likely explanation for the difficulties in production of MHC-*B*-specific alloantisera in the non-Leghorn breeds.

Immunoprecipitation and 2-D Gel Electrophoresis of Proteins

Two-dimensional gel electrophoresis distinguishes proteins based on 2 different properties: molecular mass and isoelectric point (the pH at which the protein has no electric charge). The proteins of interest (i.e., the MHC proteins) are separated from other cellular proteins by the use of specific antibodies (usually highly specific monoclonal antibodies). This immunoprecipitation product is separated by protein mass using gel electrophoresis in one direction, followed by isoelectric focusing in the second dimension. The gel is stained with a protein stain to reveal the location of the proteins specifically bound by the antibody. Both molecular mass and isoelectric point are affected by the specific amino acid composition of the protein. Variations in the protein will result in the separated proteins being located in slightly different regions on the 2-D gel, revealing whether the proteins from different individuals are the same or different.

Variation in the BG proteins of the MHC-*B* was identified using BG-specific monoclonal antibodies (Miller et al., 1984) with each MHC-*B* type studied having distinct patterns of BG proteins. This method revealed considerable structural complexity of the BG antigens. This method is not a practical method for surveying MHC diversity, as it requires a sensitive protein detection system, such as radio-isotope labeling, highly specific antibodies (monoclonal or polyclonal), and gel electrophoresis.

DNA-BASED DETECTION

Once genomic sequences became available for the chicken, the development of DNA-based detection of MHC-*B*, from diverse chicken breeds, became easier. DNA isolated from birds is easy to obtain (due to the nucleated avian red blood cells) and purified DNA can be stored and relatively easily transported across international boundaries. Detection of MHC-*B* region DNA sequence variation is repeatable on stored samples, requiring neither fresh material nor the initial development of specific antisera. The availability of DNA-based testing allowed expansion of MHC-*B* variation analysis beyond specific breeds and closed and/or inbred laboratory populations. The DNA-based detection methods used are briefly described in the following and each had their advantages and disadvantages.

Restriction fragment length polymorphisms

Early detection of DNA variation used restriction fragment length polymorphisms. Genomic DNA was digested with enzymes that cut DNA molecules at specific sequences. This digested DNA was then size-separated by gel electrophoresis and hybridized with DNA probes that identified specific genes or gene region. Variation in single bases within the DNA recognized by the DNA site-specific enzyme, or large insertions or deletions in the region recognized by the DNA probe could be detected. DNA probes that were composed of specific MHC-*B* gene sequences would bind to the digested DNA, resulting in highly repeatable and unique DNA band patterns that could be used to identify MHC-*B* haplotypes. Furthermore, by using specific gene sequences as probes, variation within different genes could be examined. Complete haplotype identification frequently required digestion with more than one restriction enzyme (Miller et al., 1988; Warner et al., 1989).

Initially, DNA from serologically defined MHC-*B* haplotypes was examined, confirming the MHC-*B* specificity as defined by serology (Miller et al., 1988; Warner et al., 1989; Lamont et al., 1990; Juul-Madsen et al., 1993). Different studies used different probes, thus identifying variability information for different genes. This method of MHC-*B* variation detection was extended to less well-studied populations, including divergently selected lines, meat-type birds, commercial broilers, and other breeds (Landesman et al., 1993; Uni et al., 1993; Zheng et al., 1999; Yonash et al., 2000; Livant et al., 2001; Emara et al., 2002; Iglesias et al., 2003).

Single-Strand Conformation Polymorphism

Single-strand conformation polymorphism (SSCP) detects small variations in single-stranded DNA fragments that cause changes in electrophoretic mobility. These DNA variants may be single base changes, or small insertions/deletions. Multiple copies of a short DNA segment are generated by the PCR process. The subsequent PCR amplicon is denatured into single-stranded DNA which folds, assuming a 3D structure unique for each specific DNA sequence. The mobility of each unique amplicon varies depending on this 3D structure. The use of PCR primers specific for genes or gene regions allows study of specific sections of the genes under interrogation. Single-strand conformation polymorphism for MHC-*B* and MHC-*Y* has been applied to examine the antigen binding region sequences (Afanassieff et al., 2001; Goto et al., 2002; Iglesias et al., 2003).

The SSCP method has been used successfully in defining MHC types within serologically defined haplotypes from the WL breed, as well as haplotypes from commercial broiler breeder hens and commercial broilers (Goto et al., 2002; Iglesias et al., 2003). Furthermore, it can identify putative MHC-*B* recombinants (Goto et al., 2002). Although SSCP typing of MHC-*B* is technically straightforward, small variations in methodology could influence the resulting patterns making it difficult for broad applications across different laboratories.

Sequence

Variation has also been examined by sequencing of short sections of several genes within the MHC-*B*. Focus
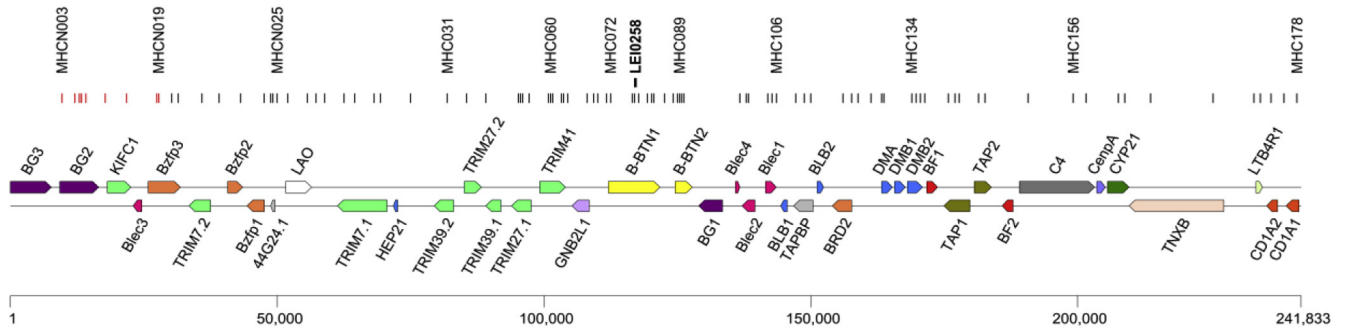
Figure 1. Gene map of the MHC-*B* region based on GenBank accession AB268588. The positions of the SNP within the MHC-B SNP panel and the LIE0258 are indicated. Those SNP indicated in black are used for the MHC haplotype designation and the red SNP are those that show evidence of gene deletion or duplication in certain haplotypes. Figure was modified from Shiina et al., 2007 and used with permission from Fulton et al., 2016b.

was placed on sequencing of coding regions (exons), and multiple variants were identified that resulted in amino acid changes in the subsequent proteins (Pharr et al., 1994, 1998; Hunt and Fulton, 1998; Livant et al., 2004). Some of these variants were common to the same gene from different haplotypes. In addition, some sequence information explained the serologically detected cross-reactivity observed among certain haplotypes (Fulton et al., 2001). Sequence-based detection of MHC variability has been successfully used with broilers (Livant et al., 2001; Lavi et al., 2005), identifying novel MHC variants within those gene regions specifically targeted. However, this sequencing approach does have limitations. Information is obtained only for the small and specific region that is being targeted, and caution must be taken to ensure that PCR-induced recombinants are not sequenced and misidentified as an actual MHC sequence. Furthermore, the process to deconvolute the sequences from MHC-*B* heterozygous samples to identify each haplotype present can be difficult. PCR fragments obtained from cDNA can be cloned into a vector and transfected into bacteria to obtain large amounts of the specific gene sequence before sequencing. This is one way to ensure that the sequence results are obtained from expressed genes. However, it greatly adds to the time and cost of identifying MHC-*B* variants.

Hosomichi et al. (2008) obtained sequences for a 59 kb region from 14 serologically defined MHC-*B* haplotypes, using multiple primer pairs and aligning the pieces. Some haplotypes were found to be identical for both amino acid and nucleotide sequences for specific genes, indicating shared gene alleles. They also found gene structural differences for multiple genes resulting in differing numbers of exons for specific genes depending on haplotype. The presence of indels resulted in coding sequence length variation of the same gene from different haplotypes. Furthermore, single nucleotide deletions revealed frame shift mutations in other genes. The detailed examination of the sequence of these 14 haplotypes provided evidence of mutation, recombination, insertions/deletions, and gene conversion events as contributing to MHC-*B* diversity.

Newer next generation sequencing methods are compatible with larger numbers of samples as multiple samples can be sequenced simultaneously. These methods provide sequence information across larger MHC-*B* segments. However, great care must be taken in ensuring that sequence primers are specific to one unique region of the MHC-*B*, which can be difficult with the multiple gene families and gene duplications known to occur within the chicken MHC-*B*. These methods require considerable up-front costs for the addition of unique bar codes to each sample, plus specialized software for data analysis.

Microsatellite markers

Microsatellite markers are very short DNA sequences (usually 1-5 bases) that are repeated many times (5-50 repeats). The LEI0258 marker is located within the MHC-*B* region (see Figure 1). Although often identified as a microsatellite marker, LEI0258 is actually a complex variable number of tandem repeats (**VNTR**) as it contains 2 different repetitive elements (12 and 13 bp), each of which can be found repeated from 1 to 30 times. There is additional variation in this marker due to small insertions and deletions within the flanking regions (Fulton et al., 2006). Over 80 alleles for LEI0258 have been identified (Chazara et al., 2013). This marker has been shown to be useful in identifying serologically defined MHC-*B* haplotypes (Lima-Rosa et al., 2005; Fulton et al., 2006). However, this marker is not sufficient to define MHC-*B* haplotypes as serologically distinct haplotypes can have identical LEI0258 allele sizes (e.g., B2 and B15 have identical LEI0258 allele size of 261 bp). One allele (357 bp) is shared in 9 different haplotypes. Thus, the sole use of this marker can underestimate MHC-*B* diversity. Furthermore, the same MHC-*B* haplotype can have different LEI0258 alleles, owing to mutation rate for the number of the 2 repetitive inserts present (Fulton et al., 2006). Because this marker examines only one point within the MHC-*B*, it is not useful in detecting recombinant haplotypes.

The availability of the LEI0258 marker as an indicator of MHC variability has allowed rapid assessments of MHC-*B* diversity. The allele size differences are generally large (12–13 bp) which can be rapidly and inexpensively visualized using low technology methods such as

agarose gel electrophoresis. The use of a DNA sequencer for more accurate allele size estimation may be required to distinguish alleles that differ due to the 1–2 bp indels that can occur in the flanking regions. The fragment sizes obtained are dependent on both the particular instrument used and the specific internal size ladder used for fragment size estimation, as size variation for the same sample on different instruments can vary from 2 to 8 bp. However, the ease of use and the variability that it can detect has resulted in the extensive use of LEI025 for identifying MHC variability. This marker has been extensively used in examining MHC-B variation worldwide in village chickens, indigenous breeds, and wild Junglefowl in multiple countries including Cameroon, China, India, Iran, Korea, Malawi, Tanzania, and Zimbabwe (Hoque et al., 2011; Izadi et al., 2011; Chazara et al., 2013; Han et al., 2013; Nikbakht et al., 2013; Ncube et al., 2014; Hako Touko et al., 2015; Mwambene et al., 2019).

Many of the earlier DNA-based detection methods had limitations in their practical application. Some required large amounts of DNA, and only limited numbers of samples could be tested, with extensive time required to obtain results. The DNA-based patterns produced were often very complex even within MHC-B homozygotes, which made analysis and interpretation difficult and time consuming, particularly for highly heterogeneous populations. The time required for analysis and the high cost of many of these tests precluded their use for routine testing needed for selection. The LEI0258 marker is amenable to higher throughput genotyping using a DNA sequence instrument which gives very accurate allele sizes. This method was used within samples from a commercial breeding program and demonstrated the association of this MHC-B marker with resistance to MDV in brown egg laying breeds (Fulton et al., 2013).

SNP Panel

Recently, a panel of SNP encompassing the MHC-B region was described (Fulton et al., 2016b). This SNP panel consists of 101 SNP, covering 230,000 bp of the MHC-B region, which includes 46 genes (see Figure 1). The individual SNP detection is based on allele-specific fluorescence labeling of the SNP alleles, using KASP chemistry (LGC, Hoddeston, UK; Semagn et al., 2014). SNP detection is not restricted to this specific technology, and any reliable SNP detection method should work. The SNP were selected based on multiple factors including wide coverage of the MHC-B region, polymorphism within the initial sample set tested, and performance reliability. Most of the SNP are located in introns or intergenic regions. Although each SNP assay provides information specifically for the polymorphic base to which it is designed, obtaining genotypes from the multiple SNP results in identification of MHC-B haplotypes, which can indirectly capture haplotype-specific variation.

Figure 1 is a gene map of the MHC-B region (published by Fulton et al., 2016b). It shows the approximate position of each SNP from the MHC-B SNP panel and the LEI0258 marker. The SNP indicated in red are those that indicate duplication/deletion and are not used for haplotype identification.

The initial description and validation of this method showed that the SNP-based haplotypes were identical for serologically defined identical haplotypes from multiple sources, confirming this as a reliable and consistent detection system. Application of the panel revealed the existence of 78 unique MHC haplotypes from multiple breeds and sources, primarily held at institutions within Canada and the United States. Topological clustering of these haplotypes based on the proportion of identical SNP genotypes resulted in the identification of 22 haplotype families, labeled A through V. This more detailed identification of MHC variation necessitated the development of a novel nomenclature to identify unique haplotypes as B serology and/or LEI0258 allele information was either not available or insufficient to unequivocally identify all 78 haplotypes. The terminology 'BSNP' was used to indicate that these haplotypes were based on SNP information within the B region only. When available, information on LEI0258 allele size and serological B type was also included. Thus the haplotype BSNP-A04 (357;B21) is a member of family A. It has the LEI0258 allele size of 357 and is the same as the B21 haplotype defined by serology.

This SNP panel was used on previously reported serologically defined MHC-B recombinants and confirmed the parental combinations that contributed to these recombinants. It also allowed finer mapping of the region within which these recombination events occurred. Furthermore, the use of this SNP panel with other samples identified additional MHC-B recombinants, provided an estimation of rate of recombination within the chicken MHC-B at approximately 7-fold higher than previously estimated by serology (Koch et al., 1983) and identified putative recombination 'hotspots' (Fulton et al., 2016b).

There is a region within the first 30,000 bp of the MHC-B (BG2 through KIFC1) that was identified by some of these SNP as being deleted or duplicated in some haplotypes. This can complicate consistent identification of the BSNP haplotypes. Subsequently, future BSNP haplotype definitions do not use this information, and thus, the MHC BSNP haplotype definitions now in use utilize 90 SNP (indicated by black lines above the genes in Figure 1, which encompasses 210,000 bp of the MHC-B).

This SNP panel has subsequently been used to examine MHC-B haplotype diversity in heritage broilers, wild Vietnamese Junglefowl, the Finnish Landrace breed, and Argentinian Campero chickens (Fulton et al., 2016a, 2017; Nguyen-Phuc et al., 2016; Iglesias et al., 2019). Additional haplotypes were identified in each new breed examined. The degree of MHC-B diversity within the wild Junglefowl was tremendous, with homozygosity being rare and complexity of heterozygote patterns causing difficulties in unequivocally identifying haplotypes. However, this SNP panel has shown its versatility in identification of haplotypes across multiple breeds.

As with other MHC detection methods, there are limitations with this SNP panel. Each SNP was chosen such that the allelic detection was consistent and reliable across multiple samples. However, genomic regions that are highly polymorphic (i.e., have multiple SNP in close proximity) are not amenable to this detection method as nearby SNP can interfere with primer binding. Thus, any SNP within highly polymorphic regions, as occurs in many genes of the MHC, would not be included. Neither can the SNP panel identify small deletions or duplications, unless they include the specific primer binding region. One case in point is that this SNP panel fails to distinguish between the 2 recombinants, BR2 and BR4, that are serologically distinguishable and known to differ by a 225 bp insertion/deletion in the BG1 untranslated region (Goto et al., 2009). It is highly likely that additional sequence diversity exists within the genome region encompassed by the panel that is not captured by the 90 SNP panel. Sequencing would be the ideal method of determining the full MHC haplotype diversity.

CONCLUSION

The application of DNA-based methods for detection of MHC-*B* variation has shown the existence of far greater MHC diversity than previously identified by the use of more traditional serological detection. The VNTR marker LEI0258 located within the MHC-*B* region has been used extensively to examine MHC diversity in multiple indigenous and wild population worldwide. But it underestimates the extant MHC-*B* variation present. The recently developed MHC-*B* SNP panel has revealed multiple novel and diverse haplotypes that were previously unknown. This SNP panel has identified gene duplication and determined that the recombination rate within the MHC-*B* is actually 7-fold higher than previously identified based on serological testing. Perhaps of most significance is the observation that there is tremendous diversity within the chicken MHC and that much of this diversity has never been studied with respect to disease resistance. With the decreasing availability of antibiotics and antiparasitic drugs for therapeutic use in commercial chickens, further investigations on the impact of MHC-*B* variation, and the genes within this important region of the chicken genome and resistance to disease, are greatly needed.

The use of DNA markers and the MHC-*B*-specific SNP panel are currently practical, repeatable, and affordable methods for identification of MHC-*B* variation. However, they cannot capture all the MHC-*B* variability that exists, that would need full sequence information. With the advent of long-read sequencing technologies, such as Oxford Nanopore Technologies or PacBio, read lengths into the 20–250 kb length can be obtained. This will allow sequence information to be obtained for potentially the entire MHC-*B* region, that is, an entire MHC haplotype, within one sequence process.

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