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Genomic Regions associated with Necrotic Enteritis Resistance in Fayoumi and White Leghorn Chickens

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ABSTRACT In this study, we used two breeds of chicken to identify genomic regions corresponding to necrotic enteritis (NE) resistance. We scanned the genomes of a resistant and susceptible line of Fayoumi and White Leghorn chickens (20 birds/line) using a chicken 60 K Illumina SNP panel. A total of 235 loci with divergently fixed alleles were identified across the genome in both breeds; particularly, several clusters of multiple loci with fixed alleles were found in five narrow regions. Moreover, consensus 15-SNP haplotypes that were shared by the resistant lines of both breeds were identified on chromosomes 3, 7 and 9. Genes responsible for NE resistance were identified in chicken lines selected for resistance and susceptibility. Annotation of the regions spanning clustered divergently fixed regions revealed a set of interesting candidate genes such as phosphoinositide-3-kinase, regulatory subunit 5, p101 (*PIK3R5*) and inositol 1,4,5-trisphosphate receptor 1 (*ITPR1*), which participate in immune response. Consensus haplotypes were found in regions containing possibly relevant genes, such as myostatin and myosin, which play important roles in muscle development. Thus, genome scans of divergent selection in multiple chicken lines and breeds can be used to identify genomic regions associated with NE resistance.

(Key words: SNP, necrotic enteritis, selected line, Fayoumi, White Leghorn)

INTRODUCTION

Necrotic enteritis (NE) is an acute *Clostridium* infection, which is characterized by severe necrosis of the intestinal mucosa (Parish, 1961). NE has recently become a significant problem for the poultry industry because of restrictions on antibiotic usage and high-density production conditions (Williams, 2005). In the United States, NE has caused enormous economic losses in the poultry industry (accounting for > $2 billion/year), largely due to the impaired growth of chickens and costs associated with medical treatments (Smith and Helm, 2008). Two common risk factors that predispose broiler chickens to NE are co-infection with *Eimeria* parasites and *C. perfringens* and a high protein diet (Park et al., 2008). Recent development of SNP genotyping arrays has enabled systematic studies of genomic variations in chickens (Groenen et al., 2011; Kranis et al., 2013). Evidence for differences in susceptibility (e.g., body weight, lesion score, and oocyst number) and gene expression data obtained by next-generation sequencing (NGS), were highly correlated in genetically different chicken lines (Jang et al., 2013; Dinh et al., 2014; Hong et al., 2014). In this study, by comparing NE susceptible and resistant lines of the Fayoumi and White Leghorn breeds, we identified the genomic regions that were divergently fixed and revealed divergent loci that are common to both genomes. The aim of our genome-wide scan was to reveal the link between phenotype and the genomic region controlling NE resistance.

MATERIALS AND METHODS

1. Animals and Genotypes

To identify the genomic regions affecting NE, two highly inbred chicken lines were examined. Two inbred White Leghorn chicken lines, which were developed by USDA-Agricultural Research Service were kindly provided by Avian Disease and Oncology Laboratory (ADOL), East Lansing, MI of A-

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griculture Research Service, United States Department of Agriculture (USDA) (Bacon et al., 2000). In addition, two highly inbred Fayoumi lines that were obtained from Iowa State University were used in our study (Zhou and Lamont, 1999). Each chicken line had been selected and maintained for decades after exposure to avian leukosis virus and Marek's disease virus. To identify NE-resistant or -susceptible lines, the body weight and lesion score were measured after oral co-infection with *Clostridium perfringens* (CP) and *Eimeria maxima* (EM). Chickens were afflicted with NE as reported previously (Jang et al., 2012). Phenotypic characteristics such as lesion score, body weight gain, and oocyst number were measured in 20~22 animals per line and genomic DNA was isolated from these animals and genotyped using a chicken 60k SNP array (Illumina, CA). From a total of 47,853 SNP loci, 235 SNPs were selected and used in all subsequent analyses after passing the SNP quality control criterion that was based on the calling rate (80%). The genome-wide SNP genotypes covered 90% of the 1,043 megabases (Mb) in the current chicken genome assembly.

2. Genetic Analysis

Considering the high levels of inbreeding, analyses based on an SNP and the corresponding haplotype were performed to examine the region associated with NE resistance. Moreover, using these analyses, SNPs carrying variant alleles in resistant and susceptible lines can be detected within a breed. For this analysis, only the completely fixed SNPs were included. Next, SNPs with divergently fixed alleles within a breed were compared between breeds. Similarly, a sliding-window analysis of the haplotype was applied to identify region(s) that might originate from a recent common ancestor. Furthermore, based on the expected length of autozygous segments (~100/2G cM, where G is the number of generations to a common ancestor) (Fisher, 1954), the size of sliding window (assumed in this case to be 20 SNPs within an ~1 Mb genomic region) is chosen to assess haplotypes that are identical by descent (IBD) and likely to originate from common ancestor (s) for up to 50 generations. Then, differently fixed haplotypes within a breed were compared with haplotypes in the other breed. Candidate regions were ordered according to the divergence between two lines that were consistent in both breeds, as well as on the basis of the haplotype analysis. Genomic inbreeding was estimated by the based on single marker homozygosity in the whole genome. In addition, genomic inbreeding was inferred based on haplotype homozygosity using the 20-SNP window.

Using Enrichr (Chen et al., 2013), we searched for the function of candidate genes interacting in pathways containing related genes. The candidate genes were selected from the regions that were differentially fixed using haplotypes in both breeds; from these, genes participating in immune response-related pathways were summarized.

RESULTS

1. Genetic Diversity and Phenotypes

None of the susceptible and resistant lines gained weight after co-infection with EM and CP; the influence of co-infection on body weight gain in the White Leghorn both line did differ from the influence of EM infection alone. In the Fayoumi resistant line, body weight gain was significantly higher in the susceptible line than in the resistant line. However, the susceptibility of the chicken lines for either breed could not be clarified based on lesion scores (Fig. S1). Although an estimation of inbreeding might depend on the definition of genomic homozygosity, we found that the chicken lines showed high levels of inbreeding (F=0.90−0.99) regardless of the methods. As expected, 99% of SNPs were fixed in the susceptible or resistant line of White Leghorn. Using a 20-SNP window, genomic homozygosity in the Leghorn lines were found to range from 0.95 to 0.96, which was higher than the levels of homozygosity measured in the Fayoumi lines (F=0.91).

2. Differentially Selected Haplotypes

Haplotype sharing on the chromosomes of susceptible and resistant lines was substantially lower than the haplotype sharing seen in Fayoumi. Using a 20-SNP sliding window analysis, 85% of haplotypes were found to be unique to the resistant or susceptible White Leghorn lines (Fig. S2). In contrast, the two Fayoumi lines shared 86% of the common haplotypes across the genome. Conversely, 14% of the total genome was considered differentially fixed by selection and inbreeding in

the two Fayoumi lines. Overall, the common candidate regions extended over 14% of the total genome, which was mostly dependent on the candidate region in the Fayoumi lines. Furthermore, the similarity of haplotypes between resistant or susceptible lines was examined between the two breeds. While comparing haplotypes, the longest common haplotype shared by Fayoumi and White Leghorn was found to not exceed 20 SNPs, suggesting that the genetic distance between the two breeds may have resulted from diversification that occurred centuries ago. Consensus 15-SNP haplotypes were detected in resistant lines on chromosomes 3, 7 and 9 (Table 1), although no common haplotypes were observed in susceptible lines. On chromosome 18, a consensus haplotype was found in susceptible lines.

3. Divergently Fixed Loci

Next, divergently fixed alleles of SNPs accounting for the difference between resistant and susceptible lines were assessed, but the differentiation was less pronounced compared to haplotype based comparisons. A total of 13,997 loci (27.7%) differentiated across the genome in White Leghorn, while alleles of 2,349 (4.6%) loci represented the difference between the two Fayoumi lines. Furthermore, 808 (1.6%) loci were common to both breeds; more specifically the same allele was fixed in resistant or susceptible lines at 223 loci, which is less than 0.5% of the total number of SNPs (Fig. S2, Table S1). One of the widely differentiated regions of the chicken genome that shows evidence of selection in two breeds occurred in 20 contiguous 20-SNP windows from 44.2 to 53.3 Mb on chromosome 3 (Fig. 1), encompassing 20 divergently fixed loci that share the identical alleles in both breeds (Table S1). Despite the presence of wide candidate regions in both breeds, our results suggest that a divergently selected region was located between 10 and 20 Mb on Chromosome 18. Other considerable candidate regions harboring a set of continuous divergently fixed loci were found in narrow regions on chromosomes 4, 12 and 14 (Table S1).

4. Genes in Candidate Regions

In addition to the regions that encompass consensus haplotypes (Table 1), five candidate regions encompassing a series of divergently fixed loci were considered for the identification of genes associated with resistance to NE (Table S1). The total size of the candidate regions was 30 Mb, which is approximately 1% of the total genome, which contains 100 known or predicted protein-coding genes. Annotation of these regions spanning clustered divergently fixed regions revealed a set of interesting candidate genes. For example, toll like receptor 3 (*TLR3*) that participates in antigen processing and presentation of peptide antigen. Seven loci were found to be divergently fixed in the interval from 62.9 to 64.6 Mb that encompasses the *TLR3* gene at 63.2 Mb on chromosome 4. It is worth noting that superoxide dismutase 2 (*SOD2*) is among the most well-supported intervals in the differential selected region of chromosome 4 (Fig. S2). *SOD2* affects multiple biological pathways including immune system development. On chromosome 18, we were able to assign the candidate region to a 5 Mb region between 4.9 and 9.8 Mb. This region contains several

Table 1. Common haplotypes in resistant or susceptible line in Fayoumi and Large White

Chromosome	Interval (Mb)	Size (kb)	Line	Genes
3	77.14～77.59	451.4	Resistant	*PRSS35, ME1, RWDD2A, PGM3, DOPEY1, UBE2CBP*
7	0.04～0.46	424.9	Resistant	*NAB1, TMEM194B, MFSD6, HIBCH, MSTN, TRNAL-CAG, MS1, ORMDL1, OSGEPL1, ASND1, SLC40A1, WRD75, COL5A2, COL3A1*
9	21.73～22.08	345.5	Resistant	*IL12A, SCHIP1, IQCJ, MFSD1, RARRES1, LXN, GFM1, MLF1, RSRC1, SHOX2*
18	6.17～6.37	199.4	Susceptible	*NOG, C18H17orf67, DGKKE, TRIM25, COIL, SCPEP1, RAB11FIP4, TRNAT-CGU, MIR1561*
18	0.50～0.71	202.9	Susceptible	*MYH6, MYH3, SCO1, C19H17orf48, TMEM220*

* Haplotype is defined by 15-SNP frame.

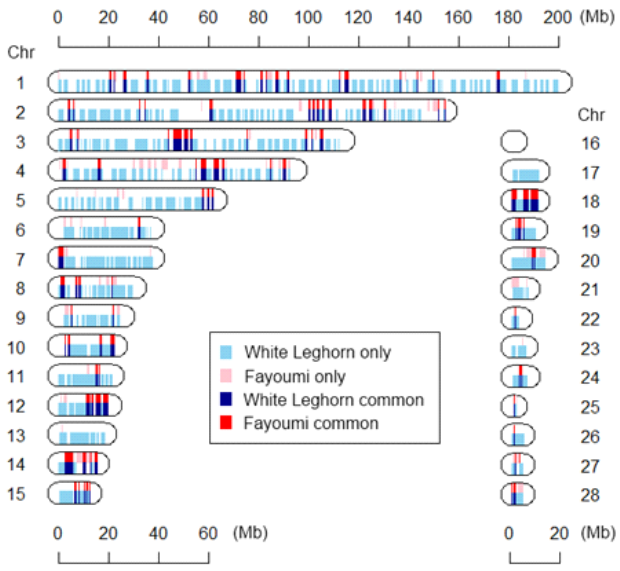

Fig. 1. Differentially selected regions in Fayoumi and White Leghorn.
Each bar represents a haplotype fragment defined by 20-SNP window. Dark blue and dark red indicate the consensus regions under differential selection in both chicken breeds. Light blue bar shows the selected regions only in White Leghorn and the selected regions in Fayoumi are shown with pink bars.

myosin gene family members, which are important in skeletal muscle production. Of particular interest are members of the myosin heavy chain on chromosome 18 (*MYH10*), which are involved in the tight junction. Interestingly, several genes (*GNA12/13*, *RAC1/3*) localized to candidate regions of chromosomes 14 and 18 are involved in regulation of the actin cytosekeleton. Although 40 genes are located in the candidate region on chromosome 12, only a few of these participate in immune response. Biological pathways in which each gene may influence NE resistance are listed in Table 2, and more detailed information is provided in Supplemental Table 2.

DISCUSSION

Despite the development of treatments for NE, genes participating in the resistance to this disease have been identified only recently. Hong et al. (2014) identified microRNA-coding genes involved in differential expression in resistant and susceptible lines. Similarly, genome-wide mapping of DNA polymorphism may facilitate the interpretation and resolution

Table 2. Functional candidate genes in the differentially selected regions

Immunological pathway	Genes in candidate regions*
Leukocyte transendotherial migration	*CANT1, NME2, NME1, TK1, POLR3K*
Cytokine cytokine receptor interaction	*PDGFRA, KDR, IL5RA, KIT*
Natural killer cell mediated cytotoxicity	*RAC3, PIK3R5*
T cell receptor signaling pathway	*CREBBP, TXK, ITPR1*
B cell receptor signaling pathway	*TEC, ITPR1*
Toll like receptor signaling pathway	*MAP2K6, PIK3R5*

* Genomic position is shown in Table S2.

of genome-wide scans of associations of traits corresponding to disease resistance. Thus, our study suggests that genome-wide scans for selecting regions associated with NE-resistance can be a useful tool when a genome-wide association study is not feasible due to limited availability of phenotype.

In White Leghorn chickens, a line was shown to be susceptible to infection by Marek's disease, whereas another line was resistant to this virus (Hong et al. 2014). Fayoumi, which originated from Egypt, is genetically distant from White Leghorn chickens. Derived from the original Fayoumi breed, the resistant and susceptible congenic pair lines are highly inbred and the two lines differ in susceptibility to *Eimeria maxima* (Kim et al. 2009). They are bred to share all genetic backgrounds, but differ in the haplotype on chromosome 16, which carries the Major Histocompatibility Complex (MHC) (Zhou and Lamont, 2003). There was no common divergent selection found in the MHC region of either breed. In this study, we found that using more chickens (N=20 per line) may not result in better resolution when considering the high levels of inbreeding.

Many of the differentially selected loci in the White Leghorn lines did not agree with those identified in the Fayoumi lines, except for 250 loci. Conversely, the number of divergently fixed alleles that were common to both breeds were decided

by Fayoumi. A comparison of the alleles within the susceptible lines of Fayoumi indicated that 236 of the 1200 SNPs were in agreement with the results obtained from the White Leghorn chickens, suggesting that the candidate regions were mostly dependent on the differential fixed alleles in Fayoumi. Furthermore, candidate regions were defined based on the density of loci that were divergently selected in both breeds. Although chicken lines were not selected for NE resistance, selection for the resistance to other infectious diseases could increase a cluster of divergently fixed SNPs around the selected loci, which may indirectly affect the resistance to NE. This analysis showed conclusively that Fayoumi and White Leghorn are homozygous for a region encompassing *MSTN*. Notably, separate mapping of both breeds revealed the same haplotypes in either resistant or susceptible lines, although most comparative haplotype analysis did not reveal a close relationship between the lines that shared the common susceptibility to NE (Table 2). Nonetheless, common haplotypes that were found in resistant lines suggest that either the resistant alleles have been introgressed from one breed to other breeds, or that the mutation is old enough to have been present in the early stages of breed separation.

We performed genome-wide analyses of selection to delimit broad genomic regions, retaining numerous candidate genes. Therefore, candidate regions were narrowed down based on the density of signals in a region, which may reflect the evidence of selection, even if selection has not been aimed for resistance to NE. When considering the history of line construction, candidate loci are likely to be associated with resistance to a specific disease, which may increase the survivability of chickens under severe conditions. Further functional evaluation of candidate genes will be required to confirm whether those genes are involved in any biological mechanism affecting resistance to NE. Six genes that have been suggested through a replication of expression (Dihn et al., 2014) are among the list of most differentially expressed genes involved in the NE resistance (Hong et al., 2014). Although experimental studies will be necessary to determine whether the genes described in Table 2 are functionally important, some genes (e.g., *SOSC3*) were identified as being differentially expressed between resistant and susceptible lines of White Leghorns (Hong et al., 2014).

Our results are unique in that they provide details of NE resistance, suggesting that the myosin gene family played an important role in the rapid differentiation of disease resistance and this may be involved in the immune mechanism. In addition, we identified numerous candidate genes that possibly contribute to breed-specific differences in susceptibility. Due to the difficultly in precisely identifying the specific gene that has been influenced by selection, we propose that a full description of divergent selection in chicken would require multiple susceptible or resistant lines to refine the region affecting NE resistance. However, this may not be a feasible approach. Thus, experimental crosses between susceptible and resistant lines will be required to refine and confirm the candidate regions that are associated with traits. Furthermore, SNPs obtained from different genetic sources will be necessary to assess those loci affecting NE resistance. Selection signature analyses do not require precise measurements of phenotypes and could be a promising approach to overcome the errors that are induced by noise in phenotypic variation and random drift (Zhang et al. 2012; Elferink et al. 2012). A deeper understanding of selection in chickens would provide important mechanistic insights into the molecular basis of short-term evolution (Rubin et al. 2010). We found that the region under selection can be obtained by comparisons between candidate regions in two chicken breeds, even if the candidate region cannot be delimited using the analysis of a single breed.

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