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No Specific Primer Can Independently Amplify the Complete Exon 2 of Chicken *BLB1* or *BLB2* Genes

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Abstract: Chicken *BLB1* and *BLB2* genes are duplicated within MHC-*B* region that plays a crucial role in disease resistance or susceptibility. To investigate the genetic polymorphism in chicken *BLB* genes, we analyzed the complete genomic DNA sequences of *BLB1* and *BLB2* genes from 14 published MHC-*B* haplotypes (59 kb). Two pairs of primers were chosen or designed to amplify the exon 2 fragments of both genes from six known MHC-*B* haplotypes. The PCR products were directly sequenced for a preliminary identification of specific variations that were further validated using cloning approach. We found that the specificity of the primers becomes ambiguous to nearly all of the MHC-*B* haplotypes. Therefore it is impossible to design specific primer according to the complete exon and intron sequences for an independent amplification of complete exon 2 of the *BLB1* or *BLB2* genes. This calls for an alternative strategy for the investigation of genetic variations in the *BLB* genes.

Key words: Chicken, MHC, *BLB1*, *BLB2*, exon 2, polymorphism

INTRODUCTION

Chicken Major Histocompatibility Complex (MHC) *BL* genes encode molecules that are similar to the classical MHC class II of its mammalian counterparts located on the surface of antigen-presenting cells including macrophages, dendritic and B cells (Davison, 2008) and involved in the antigen-presenting identification process initiated by T cells (Erf, 2004; Steinman, 2007) and also the interaction between T and B cells (Vainio *et al*., 1984; Lamont, 1989) for the development of adaptive immunity. MHC class II molecules are composed of two non-covalently associated glycoprotein chains, namely the α and β chains, each has two separate domains (α1 and α2; β1 and β2) (Lamont, 1989). The class II α chain is encoded by a low polymorphic *BLA* gene which is located 5.6 cM away from MHC-*B* region (Salomonsen *et al*., 2003). The class II β chain is encoded by *BLB1* or *BLB2* gene which are duplicated and flank the *Tapasin* gene in opposite transcriptional directions with promoters next to *Tapasin*. *BLB1* (*BLB* minor) is weakly expressed and mapped between *B-lec1* and *Tapasin* while *BLB2* (*BLB* major) is dominantly expressed and located between *Tapasin* and *RING-3* (Kaufman *et al*., 1999; Jacob *et al*., 2000). The *Tapasin* and *RING-3* were revised into *TAPBP* and *BDR2* by Shiina *et al*. (2007) based on the complete sequence of MHC-*B* region (242 kb) of a red jungle fowl, thus the locations of *BLB1* and *BLB2* were later mapped between *Blec1* and *TAPBP* and between *TAPBP* and *BDR2*, respectively.

T cell response is restricted to MHC class II-bound antigenic fragments, therefore the diversity of the MHC class II's antigen-binding region plays an important role in the initiation of the adaptive immune responses (Zekarias *et al*., 2002). A high level of polymorphisms in *BLB1* and *BLB2* exon 2 that codes for the β1 domain in the antigen-binding region (Garrigan and Edwards, 1999; Edwards *et al*., 1995) has been reported (Jacob *et al*., 2000; Goto *et al*., 2002; Hosomichi *et al*., 2008; Worley *et al*., 2008), this in turn greatly enriched the antigen types being recognized by the MHC class II molecules. Therefore *BLB1* and *BLB2* are believed to be associated with resistance or susceptibility to many diseases, such as Marek's Disease (MD) (Niikura *et al*., 2004) and salmonellosis (Liu *et al*., 2002; Zhou and Lamont, 2003). To define the influence of MHC-*B* haplotypes on disease resistance, a number of inbred and congenic lines carrying specific haplotypes were developed (Bacon *et al*., 2000). For example, Bacon and Witter (1993) used the Md5 strain of MD virus to infect

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chickens from five 15.*B*-congenic lines that were vaccinated using three serotypes of vaccines. They found that different haplotypes developed variable protective efficacies. Recently, Hosomichi *et al*. (2008) sequenced complete genomic DNAs of 14 MHC-*B* haplotypes across a region of 59 kb covering 14 genes from *BG1* to *BF2*. These sequences have provided a foundation for accurate typing such copy genes as *BLB1* and *BLB2*. Through comparative analysis of these sequences, we found that the similarity of the *BLB1* and *BLB2* Coding Sequences (CDS) of the same haplotype is as high as 95-98% and among haplotypes still around 87.5%. Among the 14 haplotypes, there are 99 mutations in the CDS and 87% of them exist within the exon 2. Goto *et al*. (2002), Xi *et al*. (2001), Xu *et al*. (2005a, 2005b, 2007), Li *et al*. (2008) and Worley *et al*. (2008) have investigated the polymorphisms of the exon 2 of *BLB* genes in different chicken and red jungle fowl populations. This paper aims to discuss the limitations of methods used for amplification of the exon 2 and future direction that can accurately identify genetic variations within the complete exon 2 of *BLB1* or *BLB2* genes.

MATERIALS AND METHODS

Samples: B2, B5, B13, B15, B19 and B21 chicken genomic DNAs were extracted from six inbred lines developed based on the identification of microsatellite variations (Fulton *et al*., 2006) by the Harbin Veterinary Research Institute, CAAS.

Primers: The sequences of primers designed by Xu *et al*. (2005a, 2005b, 2007) were as follows: F2s-up (5'-CAG CGT TCT TCT TCT GCG GT-3') and R2s-dn (5'-TCA CCT TGG GCT CCA CTG CG-3').

Based on the comparative analysis of complete *BLB1* and *BLB2* genes from the 14 MHC-*B* haplotypes (Hosomichi *et al*., 2008), we designed another pair of non-specific primers that were used to simultaneously amplify the complete exon 2 of both *BLB1* and *BLB2*: HF (5'-TGT GTG CCC TGA CCG TGC CCT-3') and HR (5'-GCA CTC ACC GCT CCT CTG CAC-3').

All the primers were synthesized and purified using ULTRAPAGE by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.

PCR conditions and procedures: All PCRs were performed with 100-200 ng of genomic DNAs in a 50 μL reaction containing 8 pmol of each primer, 2 μL (2.5 mm) dNTPs (Tiangen Biotech (Beijing) Co., Ltd.) and 25 μl of 2 x GC Buffer II incorporating 1 unit *LAX-Taq* polymerase (Beijing Huitian Dongfang Sci. and Tech. Co., Ltd.). A standard three-step thermocycling procedure applied for all amplifications was as follows: an initial denaturation of 5 min at 95°C, 35 cycles at 95°C for 30 s, 59°C for HF+HR or 61°C for F2s-up+R2s-dn for 30 s and 72°C for 1 min, followed by a final extension at 72°C for 10 min.

Direct and cloning sequencing: All PCR products were purified and sent to Beijing Sunbiotech Co., Ltd. for direct sequencing. Purified PCR products were further cloned into the pGM-T vectors (Tiangen Biotech (Beijing) Co., Ltd.) and 30 clones per haplotype were selected for sequencing.

RESULTS

Clean PCR products were obtained from all six known MHC-*B* haplotypes following the amplifications using two pairs of primers. A preliminary identification of specific variations from direct sequencing showed that two pairs of primers probably amplified the exon 2 from both *BLB1* and *BLB2*. These observations were verified and confirmed by the cloning approach. According to diagnostic mutations between the exon 2 sequences of the same haplotype and also among the haplotypes, we found that the pair of primers designed by Xu *et al*. (2005a, 2005b, 2007) indeed specifically amplified the *BLB2* exon 2 from B2, B13 and B21 haplotypes, but unexpectedly amplified the exon 2 of both *BLB1* and *BLB2* from B5, B15 and B19 haplotypes coincidently (Fig. 1, Table 1). While our newly designed primers simultaneously amplified the exon 2 of both *BLB1* and *BLB2* from all six haplotypes (Fig. 1, Table 1).

A further alignment of the F2s-up and R2s-dn primer sequences against homologous regions from the six MHC-*B* haplotypes indicated that the F2s-up mismatched to some *BLB1* or *BLB2* sequences in a number of nucleotides, for instance, it differed by six nucleotides from the *BLB1* of B2, B13 and B21 haplotypes. It has only one nucleotide different from the *BLB2* of B13 and B21 haplotypes and 1-2 nucleotides different from either *BLB1* or *BLB2* of B5 and B15 haplotypes. However, it is identical to the *BLB2* of B2 haplotype and also to both *BLB1* and *BLB2* of B19 haplotype, therefore it is expected to have only amplified the exon 2 of *BLB2* from B2, B13 and B21 haplotypes but can coincidentally amplify the both exon 2 fragments of *BLB1* and *BLB2* from B5, B15 and B19 haplotypes.

DISCUSSION

Although a number of studies attempted to identify and genotype the variations in *BLB* genes, a standard, accurate method is lacking. For example, Xi *et al*. (2001) and Li *et al*. (2008) had mistakenly used the pair of primers (In1 and Ex2b) from Zoorob *et al*. (1993) for the amplification of the exon 2 of *BLB2* alone. In fact, these primers were originally designed for the amplification of exon 2 from the *BLB*II family which included both *BLBI* and *BLBII* genes in that time (Zoorob *et al*., 1993). The *BLBI* and *BLBII* genes were later converted into *BLB1* and *BLB2* in 2004 (Miller *et al*., 2004). The forward primer (In1) and our newly designed primer (HF) are located in the conserved region among the 14 haplotypes. In1 (19 bp) is five nucleotides ahead of the

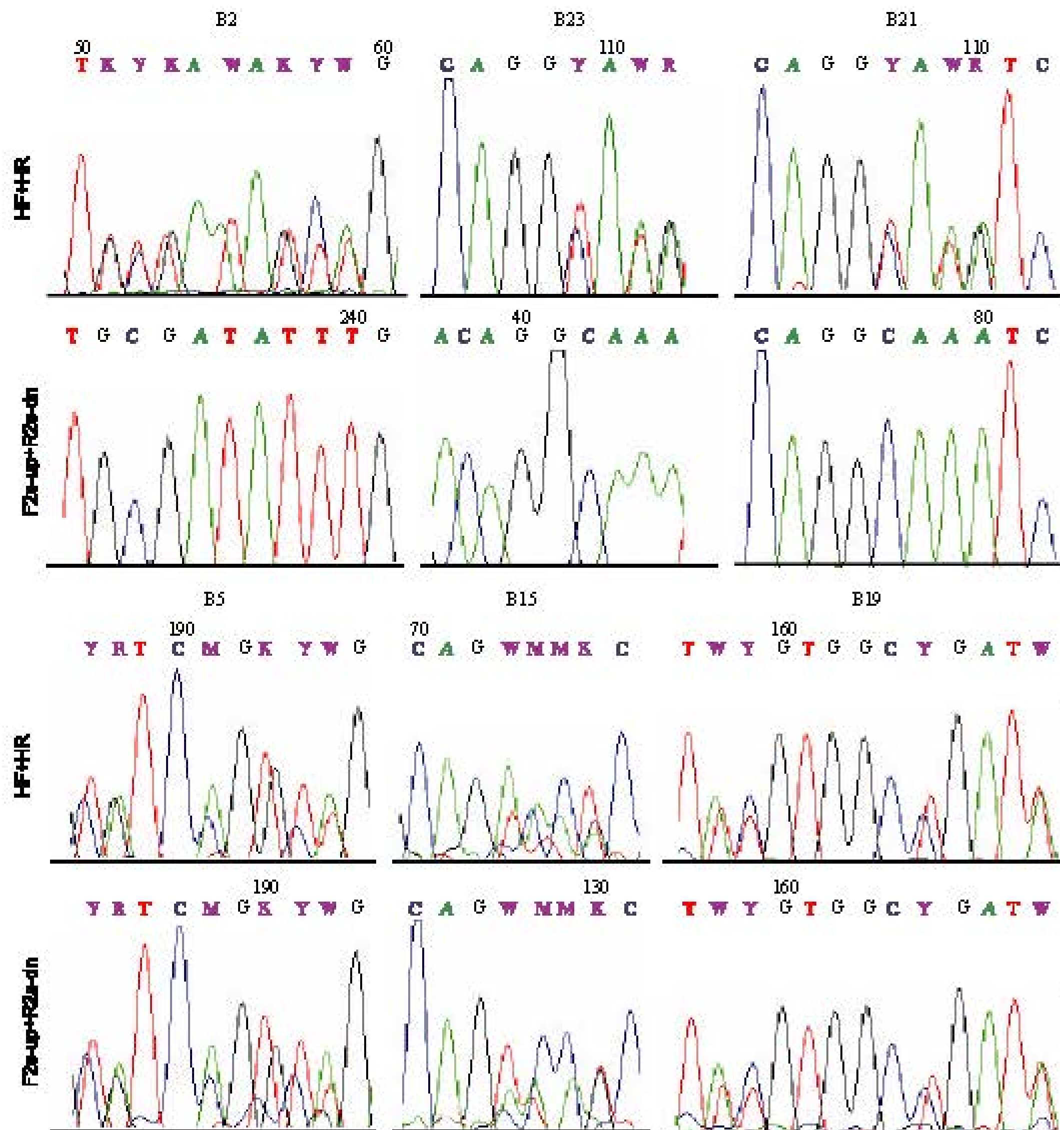

Fig. 1: The direct sequencing results from six haplotypes

Table 1: The results from cloning sequencing of BLB1 and BLB2 exon 2

	B2		B5		B13		B15		B19		B21	
Primer pair	BLB2	BLB1	BLB2	BLB1	BLB2	BLB1	BLB2	BLB1	BLB2	BLB1	BLB2	BLB1
F2s-up/R2s-dn	+	-	+	+	+	-	+	+	+	+	+	-
HF/HR	+	+	+	+	+	+	+	+	+	+	+	+

+: detected; -: not detected

HF (21 bp) and they are overlapped by 14 nucleotides, while the reverse primer (Ex2b) is located completely within the exon 2 and has only one nucleotide different from *BLB1* of B9 and B11 haplotypes as well as *BLB2* of B13 haplotype. Therefore these two pairs of primers are actually impossible to specifically amplify the *BLB2* exon 2 alone. For the pair of primers of C35 and C40 designed by Jacob *et al.* (2000), C40 is located within the exon 3. Goto *et al.* (2002) designed another pair of primers of OL284BL and RV280BL. The 18 nucleotides of RV280BL were completely covered by the Ex2b sequence (21 bp). R2s-dn (Xu *et al.*, 2005a, 2005b, 2007) is located in the conserved region between intron 2 and exon 3. In other words, similar to our HR primer, these reverse primers share the same property and almost exactly match to the homologous sequences of

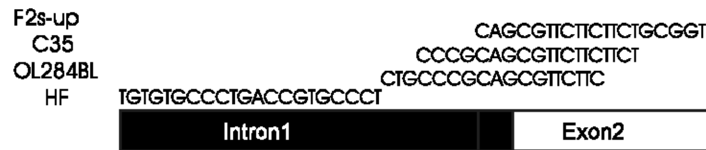

Fig. 2: The location of four forward primers

	F2s-up	C35	OL284BL	HF
BLB2-B2	CAGCGTTCTTCTTCTGCGGT	CCCGCAGCGTTCTTCTTCT	CTGCCCGCAGCGTTCTTC	TGTGTGCCCTGACCGTGCCCT
BLB1-B2	...........CAG..GAC.	...............CAG.	....	....
BLB2-B5	...............A....	....	....	....
BLB1-B5	..............GA....	..T...............G	.....T............	....
BLB2-B6	...............A....	....	....	....
BLB1-B6	...........CAG..GAC.	...............CAG.	....	....
BLB2-B8	...............A....	....	....	....
BLB1-B8	...........CAG..GAC.	...............CAG.	....	....
BLB2-B9	...............A....	....	....	....
BLB1-B9	...............A....	....	....	....
BLB2-B11	...............A....	....	....	....
BLB1-B11	...............A....	....	....	....
BLB2-B12	....	....	....	....
BLB1-B12	....	....	....	....
BLB2-B13	...............A....	....	....	.......T.............
BLB1-B13	...........CAG..GAC.	...............CAG.	....	....
BLB2-B15	..............CA....	C	....	...T.................
BLB1-B15	...............A....	....	....	....
BLB2-B17	...............A....	....	....	....
BLB1-B17	...............A....	....	....	....
BLB2-B19	....	....	....	....
BLB1-B19	....	....	....	....
BLB2-B21	...............A....	....	....	....
BLB1-B21	...........CAG..GAC.	...............CAG.	....	....
BLB2-B23	............C.C.....	................C.C	....	.......T......A......
BLB1-B23	..............GA....	..T...............G	.....T............	....
BLB2-B24	...............A....	....	....	....
BLB1-B24	...........CAG..GAC.	...............CAG.	....	....

Fig. 3: The homology between four forward primers and the *BLB*1 and *BLB*2 of 14 haplotypes (dots indicate identical nucleotides)

the 14 haplotypes, therefore we only concentrate our discussion on the locations and properties of forward primers here (Fig. 2).

C35 was designed based only on the *BLB1* and *BLB2* of B12 haplotype and it had no specificity. OL284BL was designed based on *BLB*2 from eight White Leghorn chicken sequences (B2, B4, B5, B12, B14, B15, B19 and B21), *BLB*1 from eight White Leghorn chicken sequences (B2, B4, B12, B12c, B14, B15, B19 and B21) and seven undetermined *BLB* haplotypes from commercial broiler chickens and it had eight nucleotides within the exon 2 and almost exactly matched to all of the 23 reference sequences. Our further alignment against the 14 known haplotypes detected only one nucleotide different from *BLB1* of B5 and B23 haplotypes (Fig. 3), therefore it is able to simultaneously amplify *BLB1* and *BLB2* exon 2. F2s-up was designed following only one reference sequence of *BLB2* of B12 haplotype. Compared to the homologous sequences of *BLB1* of B12 haplotype and also *BLB1* and *BLB2* of other 13 haplotypes, we found that this primer was still possible to specifically amplify the *BLB2* exon 2 of B2, B6, B8, B13, B21 and B24 haplotypes (Fig. 3). Our results supported this observation.

Therefore, we realize that it is impossible to use these primers for specific amplification of the exon 2 from *BLB2* alone if we do not know the genetic background of indigenous chickens which often carry some undefined MHC-*B* haplotypes, otherwise some *BLB1* sequences would be erroneously treated as its *BLB2* counterparts. In addition, we observe that some mutations may be covered and then missed out even if partial sequences of primers are located within the exon 2, leading to a subsequent loss of some biodiversity information of the

BLB genes. Through the analysis of sequences of all *BLB1* and *BLB2*'s introns and exons from the 14 haplotypes, we also believe that it's difficult to design specific primers to amplify the complete exon 2 from *BLB1* or *BLB2* alone. In order to obtain the complete *BLB2* exon 2 sequence, one has to use specific primers designed outside of the complete *BLB*2 genes, such as the C245 and C277 (Jacob *et al*., 2000; Worley *et al*., 2008), to amplify the *BLB2* independently, and then choose primers peripheral to the exon 2, such as the HF and HR, for direct sequencing or further secondary nested PCR (Worley *et al*., 2008).

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