Short communication: Establishment of a new polymerase chain reaction–sequence-based typing method for genotyping cattle major histocompatibility complex class II *DRB3*

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ABSTRACT

Sequence-based typing (SBT) is the most comprehensive method for characterizing major histocompatibility complex (MHC) gene polymorphisms. We report here a new PCR-SBT method for genotyping cattle MHC (BoLA) class II *DRB3* using the Assign 400ATF ver. 1.0.2.41 software (Conexio Genomics, Fremantle, Australia), which detects alleles in a semiautomated manner. We examined 12 sets of PCR reactions for their ability to amplify *BoLA-DRB3* exon 2 and selected an optimal primer set, which used ERB3N-HL031 for first-round PCR and ALL-DRB3B for second-round PCR. Next, we constructed a *BoLA-DRB3* allele database using the reference sequences of the Assign 400ATF software and successfully assigned heterozygous samples (including those with deletion alleles) using bidirectional sequencing, unlike our previously described method, which used unidirectional sequencing for detecting of deletion alleles. Next, blood samples of 128 Holstein cattle were used to correlate the results of our modified PCR-SBT method with those of our previously described PCR-SBT method. Each new PCR-SBT result corresponded completely with the *DRB3* allele that was genotyped by our previously described PCR-SBT method. Moreover, we confirmed the accuracy of our modified PCR-SBT method by genotyping 7 sire cattle and their 22 calves using Japanese Black cattle. This new method will contribute to high-throughput genotyping of *BoLA-DRB3* by sequence-based typing.

Key words: biotechnology, cattle major histocompatibility complex (BoLA) class II *DRB3* gene, PCR sequence-based typing, allele genotyping

The major histocompatibility complex (MHC) is a multigene family intrinsically involved in the adaptive immune system. The MHC class II genes encode cell surface molecules that are constitutively expressed

on antigen-presenting cells such as B-lymphocytes, macrophages, and dendritic cells. The MHC class II molecules bind and present foreign peptides to CD4⁺ T lymphocytes, and extensive research has demonstrated a high level of variation concentrated in the peptide-binding regions at several MHC loci across a wide range of species (Meyer and Thomson, 2001). The cattle MHC (BoLA) class II region consists of 1 *DRA* locus, 3 *DRB* loci, and multiple *DQA* and *DQB* genes (Takeshima and Aida, 2006). The *BoLA-DRB3* gene is the most polymorphic class II locus, which influences both the magnitude and epitope specificity of antigen-specific T-cell responses to infectious diseases. Indeed, 118 *BoLA-DRB3* alleles were reported in the Immuno Polymorphism Database (IPD)-MHC database (<http://www.ebi.ac.uk/ipd/mhc/bola/index.html>), and these alleles were associated with susceptibility of several infectious diseases such as mastitis (Sharif et al., 2000; Park et al., 2004; Rupp et al., 2007) and enzootic bovine leukemia (Juliarena et al., 2008).

Several *BoLA-DRB3* typing methods, such as those based on RFLP, PCR-RFLP, microarrays, PCR sequence-specific oligonucleotides, microsatellites, and PCR sequence-based typing (SBT), have been established (van Eijk et al., 1992; Davies et al., 1994; Sitte et al., 1996; Miltiadou et al., 2003; Park et al., 2004; Baxter et al., 2008). However, their ability to define new alleles is limited. Recently, we developed a high-resolution PCR-SBT method that recognizes the *BoLA-DRB3* alleles, including new alleles, and used 8 sequence-specific primers within the first hypervariable region (HV1) of the polymorphic exon 2 to amplify groups of *BoLA-DRB3* alleles before sequencing in large numbers of animals to be genotyped (Takeshima et al., 2001, 2002). Importantly, this strategy determined the sequence of new alleles more accurately than the PCR-SBT method described by Miltiadou et al. (2003). However, the method required 9 types of PCR reactions and 4 types of subsequent cycle sequencing reactions for each sample. Another possible weakness of this approach was that unreadable data are obtained for the first 30 bases of exon 2, including HV1, and,

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Table 1. Amplification of cattle major histocompatibility complex (BoLA) class II (*BoLA-DRB3*) exon 2 with different combinations of primers

PCR	First-round PCR		Second-round PCR		Amplification ¹	Sequence ²	Allele excluding
	Forward	Reverse	Forward	Reverse			
1	DRB3F-B	DRB3R-A	—	—	Excellent	Excellent	Excluded
2	DRB3F-B	DRB3R-B	—	—	Excellent	Dirty	NT ³
3	DRB3FRW ⁴	DRB3SBTr	—	—	Excellent	Dirty	NT
4	DRB3FRW ⁴	HL031 ⁵	—	—	Excellent	Good	NT
5	DRB3SBTf	DRB3SBTr	—	—	Excellent	Dirty	NT
6	DRB3SBTf	HL031 ⁵	—	—	Excellent	Good	NT
7	DRB3matF1	DRB3matR1	—	—	Excellent	Dirty	NT
8	DRB3matF1	DRB3matR2	—	—	Excellent	Dirty	NT
9	DRB3matF2	DRB3matR1	—	—	Excellent	Dirty	NT
10	DRB3F-B	DRB3R-A1	—	—	Excellent	Good	NT
11	DRB3F-B	DRB3R-A2	—	—	Excellent	Excellent	Excluded
12	ERB3N ⁶	HL031 ⁶	ALL ⁶	DRB3B ⁶	Excellent	Excellent	Negative

¹Excellent amplification indicates that PCR product revealed a single and specific band.

²Result of direct sequencing: dirty = not readable because of high background; good = readable but a little background detected; excellent = excellent sequencing with no background signal.

³NT = not tested.

⁴Described by Miliadou et al. (2003).

⁵Described by van Eijk et al. (1992).

⁶Described by Takeshima et al. (2001).

therefore, allele identities must be deduced from the remaining sequence data. In the present study, we improved upon our previously developed SBT method by reducing the number of PCR reactions using reselected primer sets and by using PCR-SBT analyzing software, Assign 400ATF ver. 1.0.2.41 (Conexio Genomics, Fremantle, Australia), to improve allele assignment.

To use the Assign 400ATF software for *BoLA-DRB3* PCR-SBT, we optimized the conditions for PCR amplification and sequencing, and constructed a database of *BoLA-DRB3* alleles, as shown in Figure 1A. To amplify *BoLA-DRB3* exon 2 by PCR for PCR-SBT, we synthesized 5 previously published primers and 12 newly constructed primers based on conserved regions of intron 1 or intron 2 (Table 1, Figure 1B). We carried out PCR reactions with 12 combinations of primers and obtained amplicons from all 12 primer sets, as shown in Table 1. These amplicons were sequenced by the direct sequencing method. Six of the 12 reactions failed to produce a sequencing signal, 3 reactions demonstrated good signals, and the other 3 had excellent signals. However, the *DRB3*0201* allele was excluded from 2 primer sets (sets 1 and 11). Ultimately, we selected primer pair 12, which uses ERB3N-HL031 for first-round PCR and ALL-DRB3B for second-round PCR, because it was the only primer set that was able to amplify *DRB3* region without excluding any *DRB3* allele. Next, we constructed a *BoLA-DRB3* allele database for use as the reference sequences in the Assign 400ATF software. Nucleotide sequences of *BoLA-DRB3* exon 2, positions 9 to 263, were collected from the IPD-MHC sequence

database (<http://www.ebi.ac.uk/ipd/mhc/bola/align.html>), and we used these to construct the format suitable for processing by Assign 400ATF.

The procedure for our modified PCR-SBT method for the amplification of *BoLA-DRB3* is shown in Figure 2. Genomic DNA samples were subjected to fully nested PCR, and one clear, high-intensity band of the appropriate size (355 bp) was obtained. The PCR products were sequenced directly without cloning or physical separation of the alleles, and clear heterozygous traces were obtained. The sequencing results were analyzed semiautomatically by the Assign 400ATF software. Base calling, the alignment of both strand sequences, and the building of a consensus sequence were all automatically processed. Editing of mismatched sequences was processed manually and by Assign 400ATF calling the most preferable allelic pair. When the analyzed sequence did not match the reference sequence, we continued editing manually. Ultimately, the sequence should completely match the reference sequence. If animals included new alleles or were dizygotic twins, the sequence results did not match the reference sequences. In those cases, we performed additional PCR reactions using 8 specific primer pairs, as described previously (Takeshima et al., 2001). In most cases, this step allowed us to obtain 2 separated sequences of the allele from one animal.

To confirm whether our modified *BoLA-DRB3* PCR-SBT method was accurate, we applied this method to genomic DNA extracted from whole blood of 128 Holstein cattle whose *DRB3* alleles were defined by our

Table 2. Comparison of described previously PCR-sequence-based typing (SBT) and new PCR-SBT methods

Sample ID ¹	Described previously PCR-SBT ²		New PCR-SBT ²		Sample ID ¹	Described previously PCR-SBT ²		New PCR-SBT ²	
1	1501	1101	1501	1101	65	0101	1501	0101	1501
2	14011	1001	14011	1001	66	1201	1501	1201	1501
3	0101	2703	0101	2703	67	1101	1001	1101	1001
4	1001	2703	1001	2703	68	2703	1501	2703	1501
5	0902	0902	0902	0902	69	2703	2703	2703	2703
6	0101	1501	0101	1501	70	0101	0101	0101	0101
7	0101	2703	0101	2703	71	1101	1101	1101	1101
8	0902	0902	0902	0902	72	0101	1001	0101	1001
9	0101	1201	0101	1201	73	0902	0101	0902	0101
10	1001	0101	1001	0101	74	2703	1501	2703	1501
11	1001	1201	1001	1201	75	14011	1201	14011	1201
12	0101	1501	0101	1501	76	0601	1501	0601	1501
13	1201	1501	1201	1501	77	0902	1501	0902	1501
14	1101	0101	1101	0101	78	1001	0902	1001	0902
15	0101	1201	0101	1201	79	14011	1501	14011	1501
16	1001	1101	1001	1101	80	1601	1501	1601	1501
17	1201	1601	1201	1601	81	14011	1501	14011	1501
18	1101	2201	1101	2201	82	1001	1501	1001	1501
19	1001	0101	1001	0101	83	0101	1201	0101	1201
20	0101	1501	0101	1501	84	1101	2703	1101	2703
21	0101	1501	0101	1501	85	0701	1501	0701	1501
22	1101	1001	1101	1001	86	0902	1501	0902	1501
23	2703	2703	2703	2703	87	0101	0101	0101	0101
24	0902	0101	0902	0101	88	1501	1501	1501	1501
25	0902	1201	0902	1201	89	0601	1601	0601	1601
26	1201	0201	1201	0201	90	1201	1501	1201	1501
27	14011	14011	14011	14011	91	0101	2703	0101	2703
28	0101	1701	0101	1701	92	0101	1101	0101	1101
29	0101	1501	0101	1501	93	1201	1601	1201	1601
30	2703	1501	2703	1501	94	1101	1101	1101	1101
31	1101	0101	1101	0101	95	0201	1201	0201	1201
32	1101	1501	1101	1501	96	1001	0201	1001	0201
33	1201	1201	1201	1201	97	1201	0201	1201	0201
34	1001	1001	1001	1001	98	1201	1501	1201	1501
35	0101	1201	0101	1201	99	14011	1501	14011	1501
36	1101	1601	1101	1601	100	2703	0201	2703	0201
37	1101	2703	1101	2703	101	0101	0101	0101	0101
38	1501	1501	1501	1501	102	0601	1201	0601	1201
39	1101	1701	1101	1701	103	1201	1201	1201	1201
40	1101	1201	1101	1201	104	1801	1501	1801	1501
41	1101	1101	1101	1101	105	1101	0101	1101	0101
42	1101	1501	1101	1501	106	0902	1201	0902	1201
43	1101	0101	1101	0101	107	14011	2703	14011	2703
44	1101	1001	1101	1001	108	1201	0201	1201	0201
45	1101	1201	1101	1201	109	1801	1801	1801	1801
46	0101	0701	0101	0701	110	1101	1601	1101	1601
47	1101	1501	1101	1501	111	1101	1501	1101	1501
48	14011	1201	14011	1201	112	14011	1501	14011	1501
49	0901	0901	0901	0901	113	1101	1201	1101	1201
50	1001	1701	1001	1701	114	1001	1501	1001	1501
51	1101	1201	1101	1201	115	1101	1501	1101	1501
52	1001	0201	1001	0201	116	1001	0101	1001	0101
53	0101	0101	0101	0101	117	1101	0101	1101	0101
54	0101	1001	0101	1001	118	0101	0101	0101	0101
55	1101	2703	1101	2703	119	2703	1201	2703	1201
56	1001	0201	1001	0201	120	2703	1501	2703	1501
57	0101	1601	0101	1601	121	1001	0101	1001	0101
58	1501	1501	1501	1501	122	1001	1201	1001	1201
59	1101	1501	1101	1501	123	0101	0902	0101	0902
60	1101	1501	1101	1501	124	1101	1501	1101	1501
61	1101	1501	1101	1501	125	1001	1201	1001	1201
62	0101	0101	0101	0101	126	1101	1501	1101	1501

Continued

Table 2 (Continued). Comparison of described previously PCR-sequence-based typing (SBT) and new PCR-SBT methods

Sample ID ¹	Described previously PCR-SBT ²		New PCR-SBT ²		Sample ID ¹	Described previously PCR-SBT ²		New PCR-SBT ²	
63	0902	0101	0902	0101	127	1101	1201	1101	1201
64	0902	1501	0902	1501	128	0101	0101	0101	0101

¹Blood samples of 128 Holstein cattle were collected from 4 areas of the Chiba Prefecture of Japan. High-molecular-weight DNA was prepared from the whole-blood samples using Wizard Genomic DNA Purification kits (Promega KK, Tokyo, Japan). These were examined for genotyping cattle major histocompatibility complex (BoLA) class II (*BoLA-DRB3*) by the described previously *DRB3* PCR-SBT method (Takeshima et al., 2001) and the new PCR-SBT method described in this paper.

²*BoLA-DRB3* alleles were designated according to bovine leucocyte antigen (BoLA) nomenclature committee by 4- or 5-digit numerical data.

previously described PCR-SBT method (Takeshima et al., 2001). In all cases, we were able to clearly distinguish 16 distinct *BoLA-DRB3* alleles from this group of animals (Table 2). One hundred five animals yielded typical heterozygous patterns for the *BoLA-DRB3* allele, and 23 animals (numbers 5, 8, 23, 27, 33, 34, 38, 41, 49, 53, 58, 62, 69, 70, 71, 87, 88, 94, 101, 103, 109, 118, and 128) yielded a single *DRB3* sequence. We next correlated the results of our modified PCR-SBT method with those of the previously described PCR-SBT method; each new PCR-SBT result completely corresponded with the *DRB3* allele genotyped by the previously described method.

Additionally, we confirmed the accuracy of the modified PCR-SBT by genotyping 7 sire cattle and their 22 calves (Table 3). Six alleles were detected in the 7 sire cattle, and 9 *DRB3* alleles were detected in 22 calf samples: *DRB3**1201 and *DRB3**1101, *DRB3**1501 and *DRB3**1101, *DRB3**1501 and *DRB3**1101, and *DRB3**1302 and *DRB3**1601 were detected from sires R1, R3, R5, and R6, respectively, and their sons had at least one of their sire's alleles. In these 4 sires, each of the sire's alleles was detected in at least one of their sons. Two sons (5 and 6) had *DRB3**0201, which is transmitted from sire R2, and son 12 had *DRB3**1302 transmitted from sire R4. Sire R7 had only one *DRB3* allele (*DRB3**1601), and this allele transmitted to son 22. In conclusion, there was no conflict between the results of sire genotyping and that of their calves.

The SBT system for detecting *BoLA-DRB3* alleles described here has one major advantage over previously described methods. Some *BoLA-DRB3* alleles, such as *DRB3**0201, *DRB3**3301, and *DRB3**4101, include a 3-bp deletion at nucleotide positions 193 to 195. The previously described SBT determination of *DRB3* alleles remained complicated because of the existence of these deletion alleles (Takeshima et al., 2001; Miltiadou et al., 2003; Baxter et al., 2008). In this study, our method successfully assigned the heterozygous samples that included deletion alleles using bidirectional sequencing, unlike the method described previously, which used unidirectional sequencing.

In conclusion, we report here a modified PCR-SBT method for genotyping the *BoLA-DRB3* allele using Assign 400ATF software. This method greatly decreased the effort involved in genotyping and will contribute to high-throughput genotyping of *BoLA-DRB3* by sequence-based typing.

Table 3. Results of genotyping cattle major histocompatibility complex (BoLA) class II *DRB3* (*BoLA-DRB3*) by sequence-based typing (SBT)

Calf no. ¹	Sire ¹	Sire alleles	PCR-SBT alleles ²
1	R1	*1201 *1101	*0201 *1201
2	R1		*1501 *1101
3	R1		*1201 *1601
4	R1		*1201 *0101
5	R2	*0201 *1201	*0201 *1101
6	R2		*0201 *1601
7	R3	*1501 *1101	*1302 *1501
8	R3		*1302 *1101
9	R3		*0201 *1101
10	R3		*0701 *1101
11	R3		*0201 *1501
12	R4	*1302 *1501	*1302 *1101
13	R5	*1501 *1101	*1601 *1101
14	R5		*0201 *1501
15	R5		*1001 *1101
16	R5		*1501 *1601
17	R5		*1101 *1601
18	R6	*1302 *1601	*1601 *1601
19	R6		*1101 *1601
20	R6		*0201 *1302
21	R6		*1601 *0701
22	R7	*1601 *1601	*1601 *1601

¹Blood samples of 22 calves and 7 sires were collected from a single Japanese Black farm. High-molecular-weight DNA was prepared from whole-blood samples using Wizard Genomic DNA Purification kits (Promega KK.), and these were examined for genotyping of *BoLA-DRB3* by the new *DRB3* PCR-SBT method.

²Alleles determined in the sire are shown in boldface.

Figure 2. Flowchart of the modified PCR-sequence-based typing (SBT) method for genotyping cattle major histocompatibility complex (BoLA) class II *DRB3* (*BoLA-DRB3*). Amplification of *DRB3* exon 2 was performed using a fully nested PCR reaction. Primers ERB3N and HL031 (Takeshima et al., 2001), which amplify a 300-bp fragment containing exon 2 and intron sequences, were used for the first round of amplification. The thermal cycling profile was as follows: initial denaturation for 2 min at 94°C; 20 cycles of 20 s at 94°C, 20 s at 60°C, and 20 s at 72°C; and final extension for 4 min at 72°C. A 1-μL aliquot of the first-round PCR product was used for a second round of amplification with primers DRB3ALL and DRB3B (Takeshima et al., 2001). The thermal cycling profile was as follows: initial denaturation for 2 min at 95°C; 35 cycles of 20 s at 95°C, and 20 s at 72°C; and final extension for 4 min at 72°C. Reactions for the first- and second-round PCR reactions were carried out in a 25-μL final volume containing 24 μL of 1 × rTaq buffer containing 1.5 mM MgCl₂, 120 μM dNTP, 400 nM each primer, and 1 unit of rTaq polymerase (Toyoba, Tokyo, Japan). Twenty-five microliters of the second-round PCR product was purified with an ExoSAP-IT PCR product purification kit (USB Corp., Cleveland, OH), and 2 μL of the purified product was used for the subsequent cycle sequencing step. The purified amplicon (0.5 μL) was sequenced with 10 pmol of each sequencing primer, −21M13 and M13reverse, and with reagents from the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) and 5 × sequencing buffer (Applied Biosystems). The thermal cycling profile was as follows: initial denaturation for 1 min at 96°C; 25 cycles of 20 s at 96°C, 20 s at 55°C; and 4 min at 72°C. Reaction mixtures were then subjected to automated sequencing analysis using an ABI3730 (Applied Biosystems) according to the manufacturer's instructions. Sequencing signals were analyzed by Assign 400ATF ver. 1.0.2.41 software (Conexia, Fremantle, Australia). If animals included a new allele, or were dizygotic twins, the sequence results would not match the reference sequences. In those cases, we performed additional PCR reactions using 8 specific primers described previously (Takeshima et al., 2001).

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