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Intensive DNA Sequence Characterization of Alleles at MCW0330 and LEI0094 Microsatellite Loci in Chicken

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ABSTRACT

To understand DNA sequence structural variation and its relationship with allelic distribution pattern obtained from genotyping data of microsatellite markers, Polymerase Chain Reaction (PCR) products of major alleles at two supposedly simple di-nucleotide chicken microsatellite loci of MCW0330 and LEI0094 were directly sequenced. The new sequences were compared with published data retrieved from the GenBank and Ensembl databases. The results showed that repeat unit at LEI0094 locus was a simple di-nucleotide of (AC)n for most alleles while the remaining alleles had their (AC) n irregularly interrupted by one or two (GA) nucleotides. On the other hand, MCW0330 locus carried a very complicated compound microsatellite consisted of three big structural blocks as its repeat units. These units consisted of CACAGACACA, CAGACACA and CTCAGACA. A few SNPs detected in upstream flanking sequences and specific combinations of basic structural units in repeat sequences of MCW0330 and LEI0094 loci contributed to define not only alleles different in both fragment sizes and sequence structures but also to alleles of the same fragment sizes but different in sequence structures that may lead to different peak patterns observed during genotyping exercise. Such 'cryptic' alleles of the same sizes but different in sequences can lead to an underestimated value in diversity and an ascertainment bias in interpreting microsatellite data. Therefore, an intensive characterization of DNA sequences in major microsatellite alleles derived from different genetic backgrounds is warranted to understand the evolutionary mechanism of different microsatellite DNA markers.

Key words: *Gallus gallus domestica*, simple sequence repeat, polymorphism, cryptic allele

INTRODUCTION

Microsatellite DNA is termed as simple sequence repeats (SSRs) and composed of a core repeat unit of often 1 to 10 nucleotides and unique flanking sequences. A simple example of a microsatellite is "CACACACACACACA", where the di-nucleotide motif 'CA' is repeated seven times; this can be represented as $(CA)_7$. SSRs are quite common in the genomes of higher organisms. They are highly mutable because the enzymatic process that replicates DNA each time a cell divides makes 'errors' at a relatively high rate in regard to the number of repeat units.

Therefore, numerous alleles usually occur at a microsatellite locus, with each allele differing in the number of repeat units (e.g., CA_7 vs. CA_9). A slipped-strand mispairing between the two strands of DNA double helix is considered the major force to increase or decrease one or more repeat units at nascent chains during its duplication and repair processes (Ellegren, 2004; Gholizadeh and Mianji, 2007). After examining over 100 highly dispersed (dG-dT)n. (dC-dA)n sequences in human genome, Weber (1990) realized that the sequences differed from each other both in numbers of repeats and in repeat sequence types; then divided the sequences into three categories: perfect repeat sequence, imperfect repeat sequence, and compound repeat sequence. The same researcher found that informativeness (PIC) of the perfect repeat sequence increased with increasing numbers of repeat units; PIC in the imperfect repeat sequence was lower than expected on the basis of the total numbers of repeat units and the uninterrupted CA or GT repeat units had the highest PIC regardless of the repeat sequence categories. Wintero *et al.* (1992) found that the structure, number of repeat blocks and chromosomal distribution of the (dG-dT)n. (dC-dA)n sequences in pig genome were very similar to those of human genome.

Microsatellites have been developed into widely used genetic markers because they are easy to type using a Polymerase Chain Reaction (PCR) involving primers specific to unique sequences flanking the repeat units (Weber and May, 1989) and automatic sizing of PCR products on DNA sequencing facilities. Some studies showed that microsatellite DNA sequences have a lot of interspecific and intraspecific variations. Garza *et al.* (1995) compared DNA sequences of six alleles at Mfd59 microsatellite of chimpanzees with humans and found that the differences in intraspecific allele sizes were related not only to the change in numbers and combinations of two di-nucleotide repeat units of CA and TA but also to the nucleotide replacements within TA repeat region that may lead to alleles of the same fragment sizes but different in DNA sequence structures. Fulton *et al.* (2006) and Wan *et al.* (2010) conducted a comprehensive characterization of 51 and 26 DNA sequences of an extremely polymorphic compound chicken microsatellite locus LEI0258 carrying alleles ranging from 182 base-pair (bp) to 552 bp in fragment sizes, and found very complicated combinations of two repeat units of 12 and 13 nucleotides and a number of single nucleotide polymorphisms (SNPs) and insertions/deletions (in/dels) present in the sequences of both repeat units and flanking regions that defined a few alleles sharing the same fragment sizes but different in sequence structures. Feng *et al.* (2010) found that yak, taurine and indicine cattle had different sequence characteristics present in alleles at ILSTS013, ILSTS050 and SPS115 microsatellites.

Following the applications of microsatellite DNA markers to genetic characterization of chicken genetic resources (Van Marle-Koster and Nel, 2000; Wimmers *et al.*, 2000; Rosenberg *et al.*, 2001; Hillel *et al.*, 2003; Ya-Bo *et al.*, 2006), the joint ISAG (International Society for Animal Genetics) and FAO (Food and Agriculture Organization of the United Nations) Standing Committee updated the original 25 markers and recommended 30 microsatellites including 29 markers carrying di-nucleotide and one marker with tetra-nucleotide repeat units to be used for measuring biodiversity within and genetic relationships among domestic chicken breeds (lines) or populations. This gesture is a part of the secondary guidelines for development of national management plans of farm animal genetic resources (Barker *et al.*, 1993; Hoffmann *et al.*, 2004). However, each of these microsatellites was only screened and defined by a single reference DNA sequence (Crooijmans *et al.*, 1993, 1994, 1996, 1997; Cheng *et al.*, 1995; Ruyter-Spira *et al.*, 1996, 1998; Gibbs *et al.*, 1997; McConnell *et al.*, 1999). The reference genome sequence of a red jungle fowl (*Gallus gallus*) published later contributed to the second sequences to all these microsatellites (Hillier *et al.*, 2004).

Sheng-Cheng *et al.* (2010) applied 20 out of these 30 microsatellites in the assessment of genetic diversity and relationship of three indigenous chicken populations from Papua New Guinea and found that: (1) the number of alleles per locus varied between 4 and 14; (2) a few alleles at some loci were different by a single nucleotide; (3) a few alleles at particular loci showed irregular peak patterns and (4) some loci had very different alleles or allelic groups with big gaps of more than 10 bp. In this study, conducted from September 2009 to January 2011, the authors aimed to carry out an intensive DNA sequence characterization of the LEI0094 locus which had 14 continuously distributed alleles ranging from 245 to 283 bp and the MCW0330 which had only six different alleles from 254 to 286 bp with a big gap of 10 bp between two alleles of 254 and 264 bp.

MATERIALS AND METHODS

Samples: In this study, 20 chicken samples (Table 2, 3) were selected following the results of Sheng-Cheng *et al.* (2010). All blood samples were preserved on Whatman FTA® filter papers (Whatman BioScience, Maidstone, UK) and genomic DNA was recovered following the protocol of Smith and Burgoyne (2004).

Primers: The forward PCR primer (F1) and sequencing primer (F2) of LEI0094 locus located on chicken chromosome (GGA) 4 and the forward PCR primer (F1) of MCW0330 locus mapped on GGA 17 were designed according to the extended homologous sequences of UCD001 inbred red jungle fowl (*G. gallus*; Hillier *et al.*, 2004) retrieved from the Ensembl database (Flicek *et al.*, 2010); while the sequencing primer (F1) of MCW0330 and reverse primers (R) of the two loci were obtained from the recommendations (Hoffmann *et al.*, 2004)

Table 1: The information of all primers designed and used in this study

Locus	Primer sequence (5' - 3')	*Primer position
MCW0330	GGA ATT GCT CAC GTA TGA GG (F1)	4368420-4368439
	TGG ACC TCA TCA GTC TGA CAG (F2)	4368913-4368933
	AAT GTT CTC ATA GAG TTC CTG C (R)	4369178-4369199
LEI0094	GTG TAC TTA TGC AGG TGG GAT (F1)	51608987-51609007
	GAC AAC TTC TAT TGC CAT AAC (F2)	51609075-51609075
	TCT CAC ACT GTA ACA CAG TGC (R)	51609811-51609831

*Primer positions were scored based on the Eusembl Database

Table 2: Allelic polymorphism at MCW0330 locus

		Upstream flanking region			
Allele size (bp)	Samples	-27	-11	-1	Repeat region
	G. gallus	C	C	T	$(CACAGACACA)_4$CTCAGACA$(CACAGACACA)$
	G32085	C	C	T	$(CACAGACACA)_3$CTCAGACA$(CACAGACACA)$
254	P11, V16, M20	·	·	·	$(CACAGACACA)_2$**CAGACACA**
266	M05	T	·	G	$(CACAGACACA)_4$
266	E07	·	·	·	$(CACAGACACA)_4$
266	V16	·	·	G	$(CACAGACACA)_4$
272	T57	·	·	·	$(CACAGACACA)$(CTCAGACA$CACAGACACA)_2$
272	M20	·	T	G	$(CACAGACACA)_2$**CAGACACA**CTCAGACA$(CACAGACACA)$
274	O40, T57	·	·	·	$(CACAGACACA)_3$CTCAGACA$(CACAGACACA)$
286	P11, E07, N09	·	·	G	$(CACAGACACA)_6$

Dots in the same column indicate identical bases to the two reference sequence

Table 3: Allelic polymorphism at LEI0094 locus

		Upstream flanking region		
Allele size (bp)	Samples	-32	-1	Repeat region
	G. gallus	T	A	$(AC)_{17}$
	X83246	C	T	$(AC)_{16}$
247	L80	C	A	$(AC)_{11}$
247	F17	T	A	$(AC)_2GC(AC)_8$
253	E47	T	A	$(AC)_{14}$
253	L56	T	A	$(AC)_6GC(AC)_7$
245, 251, 259, 261,	L22, M07, O24, A47, K29, F17,	C/T	A	$(AC)_{10, 13, 17\text{-}20, 22, 27}$
263, 265, 269, 279,	P34, E07, E47, E353, M07, N09			
281, 283, 287	L80, E07, L56, N09	T	A	$(AC)_{14, 15, 17}GCACGC(AC)_{11}$

(Table 1). The expected sizes of PCR products were around 240 bp for MCW0330 and 700 bp for LEI0094. It was noticed that the primer sequences included in the recommendations for genotyping MCW0330 locus were same with their original design (Crooijmans *et al*., 1997) while those for LEI0094 were redesigned to amplify a fragment larger by 73 bp than that from their original design (Gibbs *et al*., 1997).

PCR conditions and procedures: This study performed two separate PCR conditions. All PCRs were performed with 3 μL (70-100 ng μL^{-1}) DNAs in a 60 μL final reaction volume. The reaction of MCW0330 contained 10 pmol of each primer, 4 μL (2.5 mM) dNTPs (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China), 6 μL of 10×buffer and 0.8 μL (2.5 units μL^{-1}) Taq DNA polymerase (Tiangen) while the reaction of LEI0094 contained 10 pmol of each primer, 4 μL (2.5 mM) dNTPs (Tiangen) and 30 μL of 2×GC buffer I incorporated with 1 unit of LAX-Taq polymerase (Beijing Huitian Dongfang Sci. and Tech. Co., Ltd., Beijing, China). The thermocycling procedure of PCR included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 59°C for LEI0094 or 60°C for MCW0330 for 1 min and 72°C for 1 min and completed by a final extension at 72°C for 7 min.

Direct sequencing and data analysis: The PCR products were purified using the gel purification kit following instructions of the manufacturer (Beijing Sunbiotech Co., Ltd., Beijing, China) and then directly sequenced using the sequencing primers and BigDye® Terminator v3.1 Cycle Sequencing Kit on an ABI 3730 Sequence Analyzer (Applied Biosystems, Foster City, CA, USA) by the Beijing Sunbiotech Co., Ltd. The raw data was manually edited using Chromas version 1.45 and aligned with MEGA4 software (Tamura *et al*., 2007). In order to explain the structural changes of DNA sequences within the repeat units and flanking regions, we only compared and analyzed sequences that were masked by the forward and reverse primers included in the recommendations (Hoffmann *et al*., 2004).

RESULTS AND DISCUSSION

DNA sequence variation of alleles at MCW0330 locus: According to the differences of allele sizes in discontinuous distribution with one big gap of up to 10 bp and regular peak patterns observed during the genotyping of MCW0330 locus (Fig. 1), five alleles (254, 266, 272, 274 and 286 bp) of eight samples were selected for direct sequencing and analyzing their nucleotide

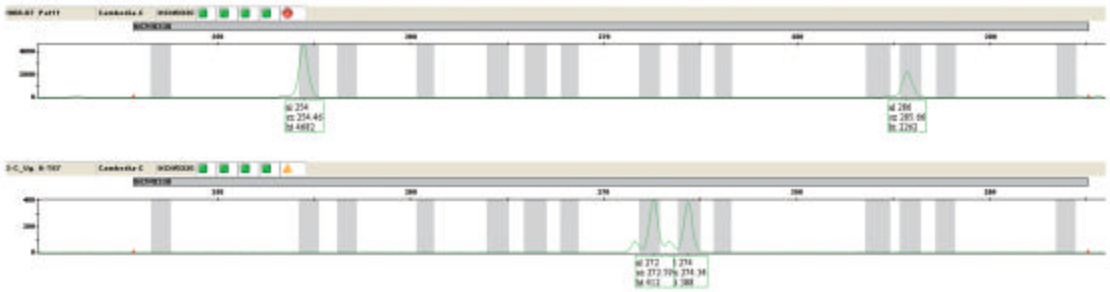

Fig. 1: The peak pattern and allelic distribution of MCW0330 locus

variations. The original single reference sequence (G32085) isolated from a White Leghorn chicken (Crooijmans *et al.*, 1993, 1997) retrieved from the GenBank database and the homologous sequence of UCD001 inbred red jungle fowl (*G. gallus*; Hillier *et al.*, 2004) retrieved from the Ensembl database (Flicek *et al.*, 2010) were included for comparison. The results showed that the upstream flanking sequence had three SNPs with a T/G transversion at -1 site present among a number of samples, a C/T transition at -11 site present only in M20 sample and a C/T transition at -27 site present only in M05 sample. The repeat unit, however, was not the expected CA di-nucleotide that was screened using synthetic poly-nucleotides of $(TG)_{13}$ (Crooijmans *et al.*, 1993, 1997). Instead it had three types of fixed structural blocks consisted of CACAGACACA, CAGACACA and CTCAGACA that can form very different and complicated combinations involving single, two or all three blocks in a particular allele, rendering MCW0330 locus a compound microsatellite (Table 2). One may argue that there is a chance of errors in generating these repeat unit sequences. However, the 15 and six sequences that were obtained using either the direct sequencing (13 and four from this study) or the cloning procedure (both two reference sequences) carried C/G and A/T transversions that were involved in constructing all three blocks. It is well known that transversion is much less common than the transition; therefore, these structural blocks were most likely the products of long-term evolution of chicken genome.

The CACAGACACA block was a primary structure and present two to six times in all alleles while the other two blocks were both minor structures and present only once separately or jointly in one each in some alleles. These unique structures with big sequence blocks further explained the limited number of alleles detected at MCW0330 locus. There was no linkage between definite SNPs, in particular the T/G transversion located at -1 site in the upstream flanking sequences and specific combinations of three blocks in the repeat sequences and also no correlation between the allelic sizes and specific combinations of three blocks. Furthermore, both SNPs in the upstream flanking region (e.g., alleles in 266 bp) and different combinations of the blocks (e.g., alleles in 272 bp) contributed to define alleles of the same fragment sizes but different in sequence structures (Table 2).

DNA sequence variation of alleles at LEI0094 locus: Based on the differences of allele sizes in nearly continuous distribution and a few irregular peak patterns observed at LEI0094 locus (Fig. 2), six alleles (247, 253, 259, 269, 279 and 287 bp) from 13 samples were selected for direct sequencing. The new sequences were aligned and compared with the original single reference sequence (X83246) that was isolated from the genomic DNAs pooled from a mixture of five

Fig. 2: The peak pattern and allelic distribution of LEI0094 locus

individuals of ISA lines and a WL female parent of the East Lansing reference pedigree following a screening using AC oligo-nucleotides (Gibbs *et al*., 1997) retrieved from the GenBank database and the homologous sequence of UCD001 inbred red jungle fowl (*G. gallus*; Hillier *et al*., 2004) retrieved from the Ensembl database (Flicek *et al*., 2010). The results indicated that most alleles at LEI0094 locus carried a repeat unit of the expected AC di-nucleotide (Gibbs *et al*., 1997), making this locus a nearly perfect microsatellite. However, there were two SNPs within the upstream flanking sequences with a T/G transversion at -1 site (T present only in the reference chicken sequence) and a T/C transition at -32 site (T and C present in each of nearly half of the alleles). The simple AC di-nucleotide repeat sequences in alleles of 247 and 253 bp were irregularly interrupted by a single GC while alleles bigger than 280 bp seem to have a fixed structure of GCACGC$(AC)_{11}$ located towards the end of repeat sequences. In addition, various combinations of SNPs in the upstream flanking sequences with different structures in repeat region (e.g., alleles in 247 bp) or different sequence structures in repeat region (e.g., alleles in 253 bp) also defined alleles of the same fragment sizes but different in sequence structures (Table 3). These different sequence structures explained the different peak patterns observed at least for alleles of 247 and 253 bp during the genotyping exercise (Fig. 2).

CONCLUSION

In this study, we attempted to validate the repeat sequence structures of two supposedly simple di-nucleotide microsatellites of MCW0330 and LEI0094 that were included in the international panel of chicken microsatellite DNA markers recommended by the ISAG/FAO Standing Committee

(Hoffmann *et al.*, 2004). However, the results of an intensive characterization of DNA sequences for major alleles at the two loci from this study were much more complicated than what we had previously thought.

Firstly, a number of novel SNPs were detected in both upstream flanking sequences of the two loci. This phenomenon was observed in both upstream and downstream flanking sequences of a mono-nucleotide microsatellite within the 16S rRNA gene in chicken mitochondrial genome (Zhao *et al.*, 2009) as well as of the LEI0258 locus within chicken MHC-B region (Fulton *et al.*, 2006; Wan *et al.*, 2010). Feng *et al.* (2010) also identified one SNP in the upstream flanking sequence among yak, taurine and indicine cattle alleles at ILSTS013 locus.

Secondly, the presence of three big structural blocks consisted of CACAGACACA, CAGACACA and CTCAGACA and their various combinations in the repeat sequences of all alleles made MCW0330 locus an unexpected, very complicated compound microsatellite with limited variations while most alleles at LEI0094 locus carrying AC repeat units proved it to be a nearly perfect microsatellite with rich variations. These findings validated a correlation between the PIC value and the perfectiveness of repeat sequences in microsatellites (Weber, 1990): the simpler and more perfect a repeat sequence is, the richer PIC and more alleles a microsatellite has.

Thirdly, both SNPs in the upstream flanking sequences and specific combinations of basic structural units in the repeat sequences of MCW0330 and LEI0094 loci contributed to define not only alleles of different fragment sizes and sequence structures but also 'cryptic' alleles of the same fragment sizes but different in sequence structures that may lead to different peak patterns observed during genotyping exercise. These observations were in agreement with the reports by Garza *et al.* (1995), Fulton *et al.* (2006), Feng *et al.* (2010) and Wan *et al.* (2010). Some microsatellites that carry such 'cryptic' alleles may show different peak patterns as what were observed in this study for LEI0094 locus only if the PCR products were sized using an automatic DNA sequencer (Fig. 2). Yet the resolution and sizing procedure applied for traditional agarose or polyacrylamide gel cannot resolve the fragments of such 'cryptic' alleles at all (Amirinia *et al.*, 2007; Qanbari *et al.*, 2007; Chatterjee *et al.*, 2008; Xin-Sheng *et al.*, 2008; Ismoyowati and Purwantini, 2010; Mahmoudi, 2010).

It is therefore particularly worth noting that misidentification of such 'cryptic' alleles can lead to an underestimated value in diversity and an ascertainment bias in interpreting microsatellite data. Therefore, intensive characterization of DNA sequences in major microsatellite alleles derived from different genetic backgrounds is warranted to improve the quality of genotyping data and to contribute to a complete understanding of the evolutionary mechanism of different microsatellite DNA markers.

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