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# A Simple Polymerase Chain Reaction-based Method for the Discrimination of Three Chicken Breeds

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**ABSTRACT :** A large number of branded chicken products exist in Japan, and in some cases, the breed of chicken is an important factor used to attract consumer interest in the retail product. In order to establish a simple method for verifying such breed claims we applied the amplified fragment length polymorphism (AFLP) technique to nine chicken breeds (White Cornish, Red Cornish, White Plymouth Rock, New Hampshire, Rhode Island Red, Barred Plymouth Rock, Hinaidori, Tosajidori, Tsushimajidori) to search for molecular markers able to discriminate chicken breeds. Three breed-specific single nucleotide polymorphisms (SNP) were identified, one for each of Hinaidori, Tosajidori, or New Hampshire. A total of 219 individuals from the nine breeds were analyzed using a specific PCR test for each of these SNP. The PCR tests made it possible to discriminate between the breeds of chickens to identify products from these three breeds. This PCR method provides an efficient method for the routine analysis and verification of certified chicken products.
(**Key Words :** Amplified Fragment Length Polymorphism, Breed Discrimination, Chicken, Polymerase Chain Reaction, Single Nucleotide Polymorphism)

## INTRODUCTION

Broiler meat produced from Western commodity breeds of chicken accounts for a large part of the chicken market in Japan. However, some specific breeds (e.g. native Japanese breeds) are used to produce branded-chicken or Jidori (area-specific chicken breeds and their crossbreds in Japan) that are arousing consumer interest. These branded-chickens or Jidoris have characteristic standards which specify the production of these chickens, for instance, the use of specific chicken breeds, feed, rearing methods, shipping age, etc. Initially these products did not have a standardized specification, and the producers of each brand established their own criteria. A specific Japanese Agricultural Standards (JAS) for naturally grown chicken (Jidoriniku) was subsequently introduced, and so far nineteen brands have been approved since 2001. The chicken breeds defined as native Japanese chicken are required to breed such specific JAS certified Jidoris. Both producers and consumers are interested in the development of reliable methods of identification and traceability of the products to retain confidence in the standards. The establishment of useful analytical methods able to ensure the origin of the products, including the breed used, will be very important in maintaining the reliability of these products in order to develop the market segment.

Amplified fragment length polymorphism (AFLP) technology is a technique for fingerprinting genomic DNA, based on the selective PCR amplification of restriction fragments (Vos et al., 1995). These fingerprints have been used as a genome mapping tool in cattle (Gorni et al., 2004), and chicken (Herbergs et al., 1999) and AFLP has been used to study genetic diversity in cattle (Ajmone-Marsan et al., 2002; Negrini et al., 2006), goats (Ajmone-Marsan et al., 2001), pigs (Foulley et al., 2006; SanCristobal et al., 2006), and chickens (De Marchi et al., 2006). This method has also been used for genetic analysis of QTL in the pig (Wimmers et al., 2002), rat (Otsen et al., 1996), and rabbit (van Haeringen et al., 2002).

The objective of this study was to establish a simple method able to discriminate between chicken breeds. We applied AFLP analysis to nine chicken breeds, White Cornish, Red Cornish, White Plymouth Rock, New Hampshire, Rhode Island Red, Barred Plymouth Rock,

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Hinaidori, Tosajidori, and Tsushimajidori, in order to identify breed-specific genetic markers. Breed-specific AFLP fragments were isolated and sequenced. Based on the sequences obtained, three breed-specific PCR assays were developed.

## MATERIALS AND METHODS

### Animals

Animals belonging to nine different breeds (White Cornish, Red Cornish, White Rock, New Hampshire, Rhode Island Red, Barred Plymouth Rock, Hinaidori, Tosajidori and Tsushimajidori) were examined in this study. The numbers of animals studied were 21 White Cornish, 22 Red Cornish, 22 White Rock, 22 New Hampshire, 20 Rhode Island Red, 20 Barred Plymouth Rock, 20 Hinaidori, 30 Tosajidori and 42 Tsushimajidori. The male:female ratio of animals was 1:1 in each breed. White Cornish, Red Cornish, White Rock and New Hampshire were bred in the National Livestock Breeding Center. Rhode Island Red, Barred Plymouth Rock, Hinaidori, 10 Tosajidori and 20 Tsushimajidori were bred in the National Institute of Livestock and Grassland Science. The remaining 20 Tosajidori were bred in the Kochi Prefectural Livestock Experiment Station and 22 Tsushimajidori were bred in the Nagasaki Prefectural Livestock Experiment Station.

### Methods

Genomic DNA was extracted from blood samples according to standard procedure (Sambrook and Russell, 2001). Individual DNA samples were mixed for each breed prior to AFLP analysis.

The AFLP core reagent kit and starter primer kit were purchased from Invitrogen Corp. (Carlsbad, CA) and AFLP analyses were performed according to the manufacturer's instructions. Selective AFLP amplification products were loaded onto a denaturing 6% polyacrylamide gel in TBE buffer and 7M urea (Amresco Inc, Solon, OH); TBE buffer was used as the electrophoresis buffer. Gels were run at constant voltage (200 V). After electrophoresis, AFLP products were transferred to nylon membrane (Roche, Basel, Switzerland) and visualized by GENOGOLD with silver enhancing (British BioCell International Ltd., Cardiff, United Kingdom).

The polymorphic bands were isolated from the gel and inserted in the pCR 2.1-TOPO vector before the transformation of TOP10 competent cells (kit TOPO TA Cloning, Invitrogen). Sequencing reactions were made with a CEQ Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter Inc, Fullerton, CA). The sequencing reaction products were read on a CEQ 8000 Genetic Analysis System (Beckman Coulter Inc). The sequences were identified in sequence searches using BLAST chicken sequences from the National Center for Biotechnology Information. To identify the polymorphic site, PCR primers were designed on the basis of sequence search results to elongate the polymorphic band. The PCR products from five individuals in each of nine breeds were sequenced and polymorphic sites were identified. Common primer pairs which amplified a target region containing a polymorphic site and primers for breed discrimination were designed on the basis of the sequenced results, and PCR was carried out on 219 individuals from nine breeds. For breed-specific single nucleotide polymorphisms (SNP), whether the SNP genotype was heterozygous or homozygous was determined with sequencing analysis in all individuals from corresponding breeds except for Hinaidori. For Hinaidori, only male samples were checked because the Hinaidori-specific SNP was positioned on chromosome Z and, in the chicken, females are the heterogametic (ZW) and males are homogametic (ZZ).

## RESULTS

Amplified fragment length polymorphism analysis of the nine breeds generated about 200 polymorphic bands in total; subsequent sequencing and PCR analysis of these bands identified three interesting breed-specific SNP with the potential to discriminate the corresponding chicken breeds. Each of the three SNP appeared to be specific for a single breed; one for Hinaidori, one for Tosajidori and one for New Hampshire (Figure 1).

Homology search results showed that the Hinaidori-specific SNP was positioned on chromosome Z, but not in a specific gene. This SNP was a nucleotide substitution from A to T at position 3015717 of chromosome Z (accession no. NW 060748). A multiplex PCR employed a common primer pair and SNP-specific primer (Table 1), and the specific primer generated the Hinaidori-specific band (Figure 1A). The SNP was detected in all Hinaidori samples analyzed (100% frequency) and was not detected in any other chicken breed investigated here (Table 2). The Tosajidori specific SNP was found to be positioned on chromosome 1 and also not in any specific gene. The SNP was a nucleotide substitution from A to G at position 1573080 of chromosome 1 (accession no. NW 060220). PCR primers were designed (Table 1) and the Tosajidori-specific band was detected (Figure 1B). This SNP was detected only in the Tosajidori samples (100% frequency) (Table 2). The New Hampshire-specific SNP was found to be located on chromosome 10 and in the guanine nucleotide exchange factor p532 gene. This SNP was a nucleotide substitution from C to G at position 19684 (accession no. NW 060433). The primers for this polymorphism (Table 1) generated a New Hampshire-specific band (Figure 1C). The frequency

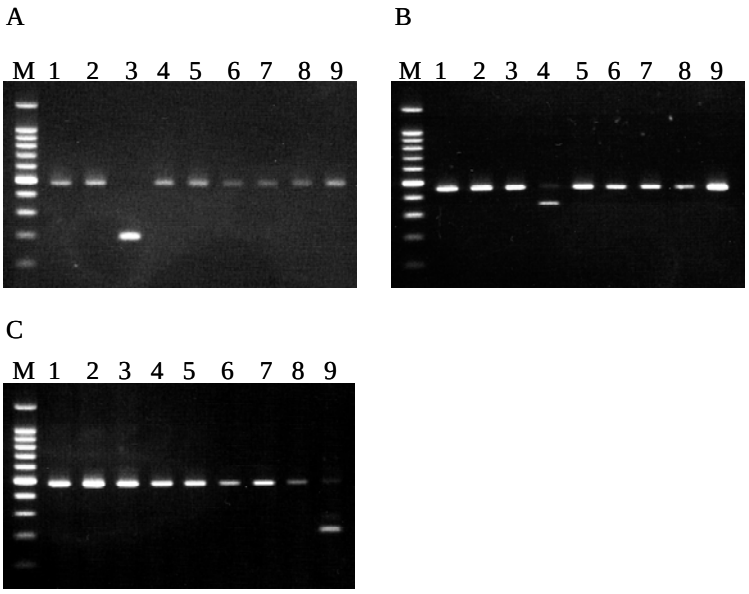


**Figure 1.** PCR amplification products using breed-specific primer and common primer pair. Lane M, size markers; lane 1, Rhode Island Red; lane 2, Barred Plymouth Rock; lane 3, Hinaidori; lane 4, Tosajidori; lane 5, Tsushimajidori; lane 6, White Cornish; lane 7, Red Cornish; lane 8, White Plymouth Rock; lane 9, New Hampshire. (A) Hinaidori-specific primer and common primer pair were used to amplify the 9 DNA samples. (B) Tosajidori-specific primer and common primer pair were used to amplify the 9 DNA samples. (C) New Hampshire-specific primer and common primer pair were used to amplify the 9 DNA samples.

of this band was 100% in New Hampshire and it was absent from all of the other breeds (Table 2). The PCR fragment sequences of the breed-specific markers are shown in Figure 2. Whether each SNP genotype was heterozygous or homozygous was not determined by this PCR analysis.

The percentage of homozygous SNP in Hinaidori, Tosajidori, and New Hampshire was 50%, 77%, and 100%, respectively (Table 3).

**Table 1.** PCR primers for the detection of breed-specific SNP

| Breeds[1] | | Sequences (5'→3') | Product size (bp) | Annealing temperature (°C) |
|---|---|---|---|---|
| H | Common primer | AGAGGTTTCTGTCTCAGCTGTG | | |
| | Common primer | TTGCTCTTCTGTTGTTTACTAATGC | 467 | |
| | Specific primer | CCAGGGCAAGATGACTGTTAA | 186 | 63 |
| TO | Common primer | AAAGAACTGGCCATTTTCAAAGC | | |
| | Common primer | AAGCAAGACTGGTTCTGGTTCTC | 444 | |
| | Specific primer | CAGCATCAACAAAAAAAGGTTAACAG | 340 | 65 |
| NH | Common primer | CTCATTAACGTTAGCAGTGAAGC | | |
| | Common primer | CAGCTACTGTTTCCAACAAAAATG | 474 | |
| | Specific primer | CTCTTTGCTTTATCCATCCAGTC | 208 | 63 |

Common primer pairs were designed to sandwich specific primers. [1] H = Hinaidori; TO = Tosajidori; NH = New Hampshire.

**Table 2.** Frequencies of the three breed-specific markers of interest

| Breeds[1] | H-specific marker | TO-specific marker | NH-specific marker |
|---|---|---|---|
| RIR | 0/20 (0%) | 0/20 (0%) | 0/20 (0%) |
| BP | 0/20 (0%) | 0/20 (0%) | 0/20 (0%) |
| H | 20/20 (100%) | 0/20 (0%) | 0/20 (0%) |
| TO | 0/30 (0%) | 30/30 (100%) | 0/30 (0%) |
| TSU | 0/42 (0%) | 0/42 (0%) | 0/42 (0%) |
| WC | 0/21 (0%) | 0/21 (0%) | 0/21 (0%) |
| RC | 0/22 (0%) | 0/22 (0%) | 0/22 (0%) |
| WR | 0/22 (0%) | 0/22 (0%) | 0/22 (0%) |
| NH | 0/22 (0%) | 0/22 (0%) | 22/22 (100%) |

[1] RIR = Rhode Island Red; BP = Barred Plymouth Rock; H = Hinaidori; TO = Tosajidori; TSU = Tsushimajidori; WC = White Cornish; RC = Red Cornish; WR = White Plymouth rock; NH = New Hampshire.


**Figure 2.** PCR fragment sequences of the breed-specific markers. The arrows indicate the positions of the PCR primers and the nucleotides in bold indicate the positions of nucleotide substitutions. H = Hinaidori; TO = Tosajidori; NH = New Hampshire.

In addition to the three breed-specific markers we also identified a number of AFLP bands that had low specificity or frequency in some breeds (Table 4). A 14 nucleotide (AAGGTGACTTAATT) deletion was identified between positions 1088645 and 1088658 of chromosome 11 and in the WD repeat domain 59 gene that was present only in Tsushimajidori but with a 50% frequency, which was therefore less useful to discriminate Tsushimajidori than the three specific markers.

**Table 3.** Frequencies of the homozygote of three breed-specific markers

| Hinaidori | Tosajidori | New Hampshire |
|---|---|---|
| 5/10 (50%) | 23/30 (77%) | 22/22 (100%) |

For Hinaidori, result from male samples was shown.

## DISCUSSION

DNA-based discrimination technologies have an advantage over biochemical discrimination methods because DNA remains a component of animal products long after they are separated from the carcass. DNA can be extracted with ease and analyzed from samples of fresh,

**Table 4.** Frequencies of the candidate for chicken breed markers with low specificity

| Breeds[1] | Candidate 1 | Candidate 2 | Candidate 3 | Candidate 4 |
|---|---|---|---|---|
| RIR | 0/20 (0%) | 20/20 (100%) | 12/20 (60%) | 0/20 (0%) |
| BP | 16/20 (80%) | 20/20 (100%) | 0/20 (0%) | 0/20 (0%) |
| H | 0/20 (0%) | 20/20 (100%) | 2/20 (10%) | 17/20 (85%) |
| TO | 0/30 (0%) | 30/30 (100%) | 11/30 (37%) | 30/30 (100%) |
| TSU | 0/42 (0%) | 21/42 (50%) | 0/42 (0%) | 1/42 (2%) |
| WC | 4/21 (19%) | 21/21 (100%) | 0/21 (0%) | 5/21 (24%) |
| RC | 1/22 (5%) | 22/22 (100%) | 0/22 (0%) | 8/22 (36%) |
| WR | 11/22 (50%) | 22/22 (100%) | 0/22 (0%) | 5/22 (23%) |
| NH | 0/22 (0%) | 22/22 (100%) | 22/22 (100%) | 0/22 (0%) |

[1] RIR = Rhode Island Red; BP = Barred Plymouth Rock; H = Hinaidori; TO = Tosajidori; TSU = Tsushimajidori; WC = White Cornish; RC = Red Cornish; WR = White Plymouth Rock; NH = New Hampshire.

frozen, or cooked animal products (Meyer et al., 1994; Tartaglia et al., 1998; Calvo et al., 2002) and thus provides a powerful means for source verification applications.

Amplified fragment length polymorphism has been adopted for the investigation of biodiversity in a wide variety of microbial, plant, and animal species. Amplified fragment length polymorphism has some advantages over other methods as a rapid, efficient, reproducible, and reliable method to scan the genome in search of specific polymorphisms. Amplified fragment length polymorphism was used to discriminate purebred and crossbred Iberian pigs (Alves et al., 2002). Strain specific AFLP markers were found in Iberian pig (Óvilo et al., 2000) and slow- or fast-growing chicken strains (Fmière et al., 2003). Breed specific markers were also found in indigenous Veneto chicken (Marchi et al., 2006).

Amplified fragment length polymorphism is considered an expensive and complex method for routine analysis. However, once AFLP has been used to identify specific polymorphisms they can be converted to simple PCR-based tests. These simple PCR reactions are then used to check for the presence or absence of these markers, thus allowing the use of these markers for large scale and sequence specific screening. It appears that this is not always successful. In attempting to convert AFLP markers to PCR markers, many of these PCR primers have either lost their specificity or their ability to amplify genomic DNA (Shan et al., 1999; Fmière et al., 2003). A possible reason for the lack of efficient conversion may lie with the nature of the AFLP polymorphisms and the method used. For example, primers may have been generated from sequences internal to the AFLP primers (as this is the simplest approach) but the nucleotide differences producing the polymorphism may have been located in the AFLP primer sequences. This specificity would be lost when internal primers are derived. Bradeen and Simon (1998) used an inverse PCR technique to characterize genomic regions adjacent to the AFLP fragments, sequence comparison of regions associated with the various alleles of interest, and the development of PCR strategies to capitalize upon genetic differences. In this case the availability of the draft genome sequence of the red jungle fowl (International Chicken Genome Sequencing Consortium, 2004) could be used to ensure that the primer sites could be considered as well as internal sequence. PCR primers were designed on the basis of the jungle fowl sequence in order to detect the breed-specific polymorphic sites. These primers successfully generated breed-specific bands for Hinaidori, Tosajidori and New Hampshire.

Alves et al. (2002) used AFLP markers to discriminate between genotypes of pigs from the perspective of protecting a brand name. Markers based on polymorphisms in coat color genes have been used to characterize pig breeds (Okumura et al., 2000; Carrión et al., 2003; Alderson and Plastow, 2004). The breed-specific SNP detected in this study may serve as an efficient tool as markers to trace origins of chicken meat, because these markers are detected only in the corresponding breed and they are found with 100% frequencies and not in any other chicken breeds. However, it will be necessary to screen larger numbers of animals for each breed to confirm these findings.

In conclusion, we used AFLP to identity breed-specific polymorphisms of chicken and developed simple PCR-based assays to detect such polymorphisms to discriminate three chicken breeds. The breed-specific markers obtained in this study are very useful because only one marker is sufficient to identify the corresponding breed without the need for complicated statistical calculation of the type required when the frequency of markers varies between breeds. In order to be able to use these markers for the verification of products then animals homozygous for the markers should be used to produce the crossbred commercial lines. Use of the breed-specific markers in this way will bring to consumers a sense of reliability required to warrant the high quality and price for this type of product.

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