◀Research Note▶

New Screening Test for Male Germline Chimeric Chickens by Polymerase Chain Reaction Using Single Nucleotide Polymorphism Detection Primers

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Since it is impossible to distinguish germline chimeric chickens by appearance only, a progeny test is required for analyzing germline chimerism. To reduce the labor, time and expense involved in such test, a new screening test for male putative germline chimeric chickens by polymerase chain reaction (PCR) was developed that uses single nucleotide polymorphism (SNP) detection primers. Putative germline chimeric chickens were produced by the transfer of stage X blastodermal cells or circulating primordial germ cells from Barred Plymouth Rocks (BPR) to White Leghorns (WL). For screening prior to the progeny test, DNA was extracted from the semen and used for PCR analysis, using SNP detection primers at position 686 in the chicken mitochondrial DNA D-loop region (DNA database, AB091008). In this study, the type of SNP in all BPR at position 686 was fixed as base G, and as base A in all WL. When the PCR product was typed as both donor (base G)- and recipient (base A)-derived, the male was determined as the germline chimera. The male and female putative germline chimeric chickens produced in this study were given the progeny test. The results of the male screening test showed good agreement with the progeny test for detecting germline chimeric chickens.

Key words : chimeric chicken, germline, mitochondrial DNA D-loop region, single nucleotide polymorphism, sperm

Introduction

Many methods have been established for producing somatic and germline chimeric chickens (for review see Petite, 2002 ; Naito, 2003). By transferring circulating primordial germ cells (cPGCs) in the bloodstream of recipient embryos, germline chimeric chickens have been efficiently produced (Naito *et al.*, 1994 ; 1998). The transfer of stage X (Eyal-Giladi and Kochav, 1987) blastodermal cells containing PGCs (or their precursors) and somatic cells into recipient embryos has produced somatic and

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germline chimeric chickens (Carsience *et al.*, 1993 ; Pettite *et al.*, 1993).

It is easy to distinguish pigmented chimeric chickens and to evaluate their percentage of donor-derived plumage. For example, when pigmented chimeric chicks are produced by the transfer of stage X blastodermal cells from Barred Plymouth Rock (BPR) to White Leghorn (WL), the donor-derived pigmentation is black. In order to test the germline chimerism of the putative germline chimeric chickens, a progeny test is usually carried out. However, this method requires much time, labor and expense. It is, therefore, necessary to develop a new and simpler method that can detect the germline chimerism of putative chimeric chickens.

The D-loop region in chicken mitochondrial DNA (mtDNA) was sequenced, and the single nucleotide polymorphisms (SNPs) were observed by Harumi *et al.* (2004), who also devised a simple method of typing SNPs by designing the primers for PCR in this region. These SNPs will serve as effective markers for detecting germline chimeras. We have developed a new screening method for detecting male germline chimeric chickens by PCR analysis before carrying out the progeny test.

Materials and Methods

Production of chimeric chickens and progeny test of female putative chimeric chickens

BPR and WL chickens are maintained as genetic closed populations at the National Institute of Livestock and Grassland Science. All animals have received humane care as outlined in the Guide for the Care and Use of Experimental Animals (National Institute of Agrobiological Sciences Animal Care Committee).

To produce chimeric chickens, donor cells from BPR were transferred to recipient embryos of WL ; the cells were collected from several embryos. Two different kinds of experiments (1 and 2) were carried out in the present study. In experiment 1, donor blastodermal cells were collected from the central disc of the area pellucida at stage X. About 500 donor cells were injected into the central disc of the area pellucida of each recipient embryo at stage X. Before the injection, to enhance the contribution of donor cells in chimeric chickens, recipient embryos were irradiated with soft X-rays for 1 min under the following conditions : 25 kV power, 5 mA, and at a distance of 18 cm.

In experiment 2, circulating primordial germ cells (cPGCs) were collected from BPR embryos at stages 13–15 (Hamburger and Hamilton, 1951). Two hundred cPGCs were injected into the bloodstream through the dorsal aorta of each WL embryo at stages 14–15 (Hamburger and Hamilton, 1951). Before the injection, to enhance the contribution of donor cells in chimeric chickens, about 8 μ l of blood were drawn from the dorsal aorta of each recipient embryo. In both experiments, after the injection of donor cells, the recipient embryos were cultured in the host eggshells until hatching, as described by Naito *et al.* (1994). The hatched putative chimeric chicks were then raised until maturity.

Both male and female mature putative chimeric chickens were mated with BPR using artificial insemination as the progeny test, and the feather color of their offspring was recorded. BPR are homozygous recessive (*i/i*) at the autosomal *I* locus, and the chick feathers are black, while WL are homozygous dominant (*I/I*), and their feathers

are white. Black offspring (*i/i*) indicate that the chicks were derived from the donor cells (BPR), while white offspring (*I/i*) confirm their derivation from the recipient cells (WL).

Detection of somatic and germline chimeric chickens by PCR

Sperm samples and blood samples were collected from the putative germline chimeric chickens, and their DNA was extracted using a DNA extraction kit (SepaGene™, Sanko Jyunyaku, Tokyo, Japan). Each 200 ng of DNA was used for PCR analysis. The PCR reaction was performed using a programmable thermal controller (Model 9700, Perkin Elmer, Shelton, CT, USA).

There is one single nucleotide polymorphism (SNP) at position 686 in the chicken mtDNA D-loop region (DNA database, AB091008). Harumi *et al.* (2004) designed the SNP detection primer sets at this position. The pair of primers typing base A are 5'-TGG GGC TTC TTC ACA GGT CA-3' and 5'-CGA CAA GCA TTC ACT AAA TAG CAC C-3', while those typing base G are 5'-CCC ATA ATA CGG CGA AGG ATT AGA C-3' and 5'-TCG CAC CCG CAC TGT GAA GGC C-3'. The PCR was performed in a 50 μ l reaction mixture containing 200 ng of genomic DNA (containing mtDNA), 5 μ l of 10 $\times$ PCR buffer, 4 μ l of dNTPs (2.5 mM each), 25 pmols of each primer, and 2.5 units of *Taq* polymerase (TaKaRa Taq™, Takara Shuzo, Shiga, Japan). After an initial denaturation step at 94°C for 2 min, 40 cycles of amplification were performed; DNA was denatured at 94°C for 30 seconds, annealed at 62°C for 30 seconds, and extended at 72°C for 1 min. The reactions were then incubated at 72°C for 2 min. The type of SNP at position 686 in all BPR chickens in this study was fixed as base G, and as base A in all WL. The germline chimeric chickens examined in this study should, therefore, be detected by mixing both bases, A and G. For internal control, a sequence of the endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified to ensure the presence of genomic DNA, using the primers designed by Love *et al.* (1994). The PCR conditions for GAPDH gene have been described by Sano *et al.* (2003).

Results

The hatched putative germline chimeric chicks were raised until maturity. In experiment 1, 13 males and 14 females were raised. Since one of the 13 males and one of the 14 females were sterile, they were both excluded from the screening and progeny tests. In experiment 2, there were 32 mature males and 13 mature females.

The male putative germline chimeric chickens were screened by PCR prior to the progeny test. The numbers of male germline chimeras detected in experiments 1 and 2 were 5/12 (41.7%) and 31/32 (96.9%), respectively (Table 1). Some of the results of the PCR analysis are shown in Fig. 1. Four and 5 putative male germline chimeric chickens produced by experiments 1 and 2 were given the progeny test, and the results are shown in Table 2. The PCR-positive chickens produced donor-derived offspring at a percentage of 2.9–19.1% in experiment 1 and 5.0–30.9% in experiment 2, whereas the PCR-negative chickens produced no donor-derived offspring.

The female putative germline chimeric chickens were given the progeny test. The

Fig. 1. The typing of SNP at position 686 in the chicken mitochondrial DNA D-loop region by PCR analysis. In this study, the type of SNP was fixed as base G in all BPR, and base A in all WL. Therefore, the chimeric chickens examined in this study must be detected by mixing both bases G and A. Lanes 1 and 9 : size marker ; Lane 2 : negative control (DW) ; Lane 3 : donor embryo (BPR) ; Lane 4 : recipient embryo (WL) ; Lanes 5 to 8 : male putative germline chimeric chickens. The three males (Lanes 5 to 7) were detected as germline chimera, since their PCR products were typed both G and A.

Table 1. Chimerism in germline and somatic cells

Cell line	Experiment 1	Experiment 2
Germline		
Males by PCR analysis	5/12 (41.7%)	31/32 (96.9%)
Females by progeny test	3/13 (23.1%)	9/13 (69.2%)
% of donor-derived offspring	0.0–30.1%	0.0–13.4%
Somatic cells		
Blood by PCR analysis	8/27 (29.6%)	0/41 (0.0%)
Pigmentation by appearance	22/27 (81.5%)	0/45 (0.0%)

numbers of female germline chimeras detected in experiments 1 and 2 were 3/13 (23.1%) and 9/13 (69.2%), respectively. The percentages of donor-derived offspring were 0.0–30.1% in experiment 1 and 0.0–13.4% in experiment 2 (Table 1).

The pigmented chimeras produced in experiment 1 were identified at hatching by the presence of black down derived from BPR. Twenty-two of 27 (81.5%) chickens raised were identified as pigmented chimeras. The percentage of donor-derived plumage was 0.0–90.0%. It was also revealed that 8 of 27 (29.6%) chickens were blood cell line

chimeras. No pigmented or blood cell chimeric chickens were detected in experiment 2.

Discussion

To produce chimeric chickens, stage X blastodermal cells or cPGCs were transferred from BPR to WL. The male putative germline chimeric chickens were screened prior to the progeny test, while both the males and females were given the progeny test. The results of the male screening test showed good agreement with the progeny test. In experiment 1, stage X blastodermal cells were transferred to stage X embryos. In experiment 2, cPGCs collected from stages 13–14 embryos were transferred to stages 14–15 embryos. Blastodermal cells at stage X contain both somatic and germline cells. Generally, the efficiency of producing germline chimeras is higher by transferring cPGCs rather than blastodermal cells (Naito, 2003). In this study, the efficiencies of producing germline chimeras in experiment 2 were higher than those in experiment 1. These results showed that the new method developed in this study should provide to be effective for screening male putative germline chimeric chickens prior to progeny testing.

The genomic DNA extracted from somatic cells contains mtDNA. Therefore, with the same PCR analysis used for the screening test of male germline chimeras in this study, one can distinguish the blood cell chimeras as well as the pigmented ones (Table 1).

The method developed in this study is effective screening for germline chimerism as a means of qualitative analysis. However, since it is difficult to evaluate the percentage of donor-derived cells by this method, it must be further improved to serve as a means of quantitative analysis. The typing of SNP is another issue still to be addressed. In this study, the type of SNP at position 686 in the mtDNA D-loop region was fixed as base G in all BPR, and as base A in all WL. However, we did not examine the SNP typing in other chicken populations. This makes it necessary to check the type of SNP in all

Table 2. Progeny test of male putative germline chimeric chickens

Experiment	Bird No.	PCR*	Black feather (%)	Number of chicks hatched	Number of white chicks	Number of black chicks	Black chicks (%)
Experiment 1	1	+	65	94	76	18	19.1
	2	+	45	160	130	30	18.8
	3	+	40	34	33	1	2.9
	4	—	40	101	101	0	0.0
Experiment 2	1	+	0	123	85	38	30.9
	2	+	0	123	98	25	20.3
	3	+	0	20	19	1	5.0
	4	+	0	37	32	5	13.5
	5	—	0	214	214	0	0.0

*“+” indicates detection of both BPR and WL-derived DNA, and “—” indicates detection of WL-derived DNA only, in the sperm samples by PCR analysis.

donors and recipients, before chimeric chickens are produced in a different laboratory. For female putative germline chimeric chickens, new screening methods prior to progeny tests must be developed.

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