Mini and Microsatellite DNA Analyses of the Irradiated Sperm Transformed Chickens

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We analysed our irradiated sperm transformed (IST) chicken DNA for minisatellite loci by DNA fingerprinting and the microsatellite DNA by PCR method.

The direct coordination between brownish egg shell color and DNA fingerprint bands was not recognized under this study condition. The genetic similarities of WL-r5 (IST line), WL-G (recipient line) and RIR-Y8 (donor line) by using band sharing (*BS*) values were calculated from the number of DNA fingerprint bands derived from mixed DNA. The value of *BS* between WL-r5 and WL-G was 0.61 in *Hae*III digestion bands and 0.48 in *Hinf*I digestion bands. The *BS* value between WL-r5 and RIR-Y8 was 0.26 in *Hae*III digestion bands and 0.15 in *Hinf*I digestion bands. Comparison of the DNA fingerprint of mixed DNA for each strain was made and the common band in both WL-r5 and RIR-Y8 was detected. This band was not detected in WL-G. Strain specific band was detected from DNA fingerprint used in mixed DNA. The major band was common in WL-r5 and WL-G. In PCR analysis, the common band in both WL-r5 and RIR-Y8 was found. From these results, it was thought that the WL-r5 genome was, for the most part, common with the recipient WL-G genome, except for a few genome from RIR-Y8 donor DNA. We thought that a few parts of donor DNA in WL-r5 were transferred through the irradiated sperm of donor RIR-Y8 male under successive selections for the brownish egg shell color from generation to generation.

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Key words : genetically transformed chicken, minisatellite DNA, microsatellite DNA, PCR, DNA fingerprint

Introduction

In our previous study, we succeeded in producing irradiated sperm transformed (IST) chickens by the use of double artificial inseminations of γ -ray irradiated donor sperm and unirradiated normal recipient sperm (YAMAMOTO and TOMITA, 1992). In that study, the resulting genetic factors responsible for the donor brownish egg shell color was 3.8% in the females of the first generation. It was suggested that the IST chickens produced by this technique integrated the fragmented donor DNA which contained the egg shell color gene(s) from the irradiated sperm. Then we tried to detect the DNA fragment from the irradiated donor sperm by DNA fingerprinting. It was revealed that 26.7% of embryos had foreign bands which were not possessed by the recipient

parent (YAMAMOTO and TOMITA, 1993).

In this study, attempts were made to detect the DNA from donor in the selected IST chicken line at minisatellite loci by DNA fingerprinting. DNA fingerprint analysis has been used for individual discrimination (JEFFREYS *et al.*, 1985 a, 1985 b, 1985 c), linkage estimation (JEFFREYS *et al.*, 1987 a) and estimation of genetic relationship (KUHNLEIN *et al.*, 1989). In addition, we compared the relationship between donor characteristic bands in IST chicken females and egg shell color variations. The genetic relationship between IST, donor and recipient chicken lines was analysed using DNA fingerprinting patterns. The typing of tandemly repeated hypervariable microsatellite loci was made by the polymerase chain reaction technique (PCR : SAIKI *et al.*, 1985).

Materials and Methods

Chickens

In this study, the following adult chicken lines were used ; White Leghorn G (WL-G, males 5, females 5) as recipient line, Rhode Island Red Y 8 (RIR-Y 8, males 6, females 6) as donor line, and White Leghorn r5 (WL-r5, males 10, females 10) as irradiated sperm transformed (IST) line. All chickens were maintained at the Poultry Research Institute, Aichi-ken Agricultural Research Center, Aichi, Japan.

Blood samples

Blood samples were taken from the wing vein with heparine sodium salt as anticoagulant. Samples were washed twice with saline and centrifuged.

DNA extraction and digestion

DNA was prepared from whole blood samples as described by CIULIA *et al.*, (1988) and MANIATIS *et al.*, (1989). DNA samples thus extracted were digested overnight with 10 unit of *Hae*III or *Hin*FI (Nippon Gene)/ μ g under the conditions recommended by the suppliers. Before loading onto the gel, all DNA samples were extracted twice with phenol/chloroform (1 : 1), once with chloroform, precipitated with ethanol, and dissolved in distilled water.

Electrophoresis and Southern blotting

After restriction endonuclease digestion, the DNA was fractionated by electrophoresis in a 1.0% agarose gel of 40 cm long, in $1\times$ TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at a constant voltage (5 V/cm). Electrophoresis was carried out at room temperature for 48 hours in nonrecirculating buffer tanks. To prevent pH change, the buffer was renewed every 12 hours. After electrophoresis, the gel was stained with ethidium bromide solution and photographed under ultraviolet (UV) light. DNA in gel was nicked with 500 ml of 250 mM HCl for 30 min, denatured with 500 ml of 1.5 M NaCl, 0.5 M NaOH for 30 min, and neutralized with 500 ml of 1.5 M NaCl, 0.5 M Tris-HCl solution (pH 7.5) for 30 min. DNA was transferred from the gel onto a nylon hybridization membrane (Hybond-N+, Amersham) by capillary Southern blotting (SOUTHERN, 1975) in a $20\times$ SSC ($1\times$ SSC : 0.15 M NaCl and 0.015 M Trisodium citrate) overnight. The DNA was fixed by soaking with 0.4 M NaOH for 2 min. The membrane was rinsed with a $5\times$ SSC solution for one min.

Hybridization

Membranes were hybridized with bacteriophage M13 mp18 RFI DNA (7249 bp, TAKARA) labelled by the nonradioisotopically enhanced chemiluminescence (ECL) method (Amersham) at 42°C overnight. The M13 probe DNA has tandem repeats of 15 bp consensus sequence (GAGGGTGGXGGXTCT) in the gene coding for the protein III region (VASSART *et al.*, 1987; RYSKOV *et al.*, 1988; GATEI *et al.*, 1991). The ECL gene detection system kit (Amersham) was used according to manufacturer's protocol. Membrane washing was carried out by two steps. First washing was carried out once with 0.1% sodium dodecyl sulfate (SDS) and 0.5×SSC at room temperature for 20 min. The second washing was done twice with the 1×SSC solution at room temperature for 5 min. Membranes were exposed to the autoradiography film (Hyperfilm-ECL, Amersham) for 1 hour at room temperature.

PCR analysis

Genomic DNA was diluted at 10 ng/μl. Fifty ng of template DNA was amplified by VNTR (variable number of tandem repeat) Fingerprinting system (Biotech International). This system was used for human individual typing based upon a hypervariable region at the 3' end of the apolipoprotein B gene (Apo B, BEORWINKLE *et al.*, 1989). Modified PCR method was used according to the manufacturer's protocol. The sequence of the 5' oligonucleotide used to prime the PCR was 5'-ATGGAAACGGA-GAAATTATG-3'. The sequence of the 3' PCR primer was 5'-CCTTCTCACTTG-GCAAATAC-3'. Initial incubation of denaturation was at 94°C for 5 min and 58°C for 6 min, followed by 38 cycles of denaturation at 94°C for 1 min, annealing and polymerization at 58°C for 6 min. Final incubation was carried out at 94°C for 1 min, 58°C for 15 min and 25°C for 5 min. The 10 μl of PCR products were electrophoresed in a 3% agarose gel at 100 V constant voltage in 1×TAE running buffer and detected by staining with ethidium bromide solution under UV light.

BS values

To assess the genetic similarity of DNA fingerprint bands between three chicken lines, the proportion of band sharing (BS) value was calculated as :

$$BSab = 2(Nab)/(Na + Nb), \dots\dots\dots(1)$$

where *BSab* = level of band sharing between lines a and b, *Nab*=number of bands shared by line a and b, *Na* and *Nb*=total number of bands for line a and line b, respectively (JEFFREYS and MORTON, 1987b; WETTON *et al.*, 1987; DUNNINGTON *et al.*, 1990). Analysis of DNA fingerprints was carried out on DNA fragments larger than about 4 kb.

Egg shell color values

Photometric measurements of egg shell color at 180 days were carried out with a reflection photo difference meter (TC-1500MC, Tokyo Denshoku, brightness : L-value, red component : a-value and yellow component : b-value) at the blunt end of the egg. For the synthetic estimates of egg shell color, Z values were calculated for each egg by a discriminate function formula using three color values (YAMAMOTO and TOMITA, 1992).

Results

Genetic similarities among three lines using band sharing (*BS*) value are shown in Table 1. Mixed DNAs of each line were used. The *BS* value between WL-*r*5 and WL-G digested with *Hae*III was calculated as 0.61 according to formula (1). The *BS* value were 0.26 and 0.24 between WL-*r*5 and RIR-Y8, WL-G and RIR-Y8, respectively. The *BS* values digested with *Hinf*I was 0.48 between WL-*r*5 and WL-G, 0.15 between WL-*r*5 and RIR-Y8 and 0.19 between WL-G and RIR-Y8, respectively.

The band of WL-*r*5 common to RIR-Y8 was found in DNA fingerprint of mixed DNA digested with *Hinf*I (Fig. 1-A). This common band appeared at about 7.0 *kb* (indicated by closed triangle). Moreover, this common band was found in six (A), (B), (C), (G), (I) and (J) of ten WL-*r*5 females (Fig. 1-B) and two (852 and 849) of ten WL-*r*5 males (Fig. 1-C) used *Hinf*I digested individual DNA fingerprint. The data were shown for only two females (G and I) in Fig. 1-B.

Fig. 2 shows line characteristic bands pattern using mixed DNA digested with *Hinf*I enzyme. WL-*r*5 and WL-G lines possessed a common band (the arrow indicate, about 21.0 *kb*) in a major band. The minor bands appeared to be line characteristic and were expressed strongly in male. RIR-Y8 line showed different banding pattern as compared with WL-*r*5 and WL-G lines.

The results of PCR analysis are shown in Fig. 3. The line characteristic band (the arrow indicate, about 2100 *bp*) was detected by electrophoresis of amplified PCR products by the primer of the human apolipoprotein B (Apo B) gene 3' hypervariable region. All WL-*r*5 males and females used in this study had this specific band. In the RIR-Y8 line, some chickens were positive in having this band and others were not. WL-G chickens did not have this band.

Photometric values of egg shell color were measured for the three components (L-value, brightness; a-value, red component, and; b-value, yellow component) in ten females of WL-*r*5 line. For example, the dark or brown shell color like Rhode Island Red eggs gave a low L-value, high a-value and high b-value compared with those of the pure white shell color like White Leghorn eggs. As shown in Table 2, the egg shell color values if WL-*r*5 (A) to (J) females varied gradually. The (A) female laid the most brownish dark color shell eggs (L-value, 75.0; a-value, 3.7, and; b-value, 18.1) in WL-*r*5 females at 180 days, and the (J) female laid the most whitish light color shell eggs (L-value, 82.4; a-value, 0.5, and; b-value, 11.7). The means of three values for WL-*r*5

Table 1. Comparison of genetic similarity between WL-*r*5, WL-G and RIR-Y8 lines of chickens using band sharing (*BS*) values of DNA fingerprinting detected by mixed DNA and probe M13 phage DNA

Line		<i>Hae</i> III				<i>Hin</i> fl			
<i>a</i>	<i>b</i>	<i>Na</i>	<i>Nb</i>	<i>Nab</i>	<i>BS</i>	<i>Na</i>	<i>Nb</i>	<i>Nab</i>	<i>BS</i>
WL- <i>r</i> 5	WL-G	99	103	62	0.61	87	95	44	0.48
WL- <i>r</i> 5	RIR-Y8	99	99	26	0.26	87	98	14	0.15
WL-G	RIR-Y8	103	99	24	0.24	95	98	18	0.19

Fig. 1. Comparison of the WL-r5, WL-G and RIR-Y8 DNA fingerprints. A : The common band found in WL-r5 and RIR-Y8 lines. Mixed DNA was used. B : The common band found in WL-r5 females. Individuals DNA was used. C : The common band found in WL-r5 and RIR-Y8 males. Each DNA was digested with *Hinf*I and hybridized with M13. F : female, M : male, ► : common band.

Fig. 2. Line characteristic band pattern of RIR-Y8, WL-G and WL-r5 lines. Mixed DNA was used after digestion with *HinfI* and hybridized with M13. →: characteristic band in WL-r5 and WL-G. F: female, M: male.

Fig. 3. PCR analysis of microsatellite DNA of the WL-r5, WL-G and RIR-Y8 lines. The primer for detecting human apolipoprotein B gene 3' hypervariable region was used. →: characteristic band in WL-r5 and RIR-Y8. M: male, F: female.

line (L-value, 78.1 ± 2.3 ; a-value, 2.0 ± 0.9 , and; b-value, 15.1 ± 1.9) were shown to be intermediate between WL-G and RIR-Y8 lines. We attempted to compare the Z value using three color values and DNA fingerprint bands, but there was no relationship

Table 2. Photometric and Z values of egg shell color of WL-r5 females at 180 days and the means of three color values of WL-r5, WL-G and RIR-Y8 lines with the number of genetically transformed bands detected in DNA fingerprint digested with *Hin* fl

WL-r5 females	No. of eggs	L-value (brightness)	a-value (red component)	b-value (yellow component)	Z value	No. of transgenic band [*]
446 (A)	1	75.0	3.7	18.1	-0.64	1
445 (B)	1	76.0	2.8	17.4	-0.42	1
439 (C)	1	74.4	3.2	17.4	-0.39	1
436 (D)	1	77.6	2.0	16.9	-0.28	0
437 (E)	1	76.2	3.0	15.3	-0.11	0
438 (F)	1	78.1	1.8	14.2	0.05	0
443 (G)	1	79.8	1.2	14.7	0.17	1
442 (H)	1	80.1	1.0	13.4	0.36	0
444 (I)	1	81.7	1.1	11.8	0.54	1
447 (J)	1	82.4	0.5	11.7	0.63	1
WL-r5	10	78.1±2.3*	2.0±0.9	15.1±1.9	—	—
WL-G	549	87.9±1.0	-1.6±0.3	5.2±1.8	—	—
RIR-Y8	147	61.5±3.3	5.3±0.8	18.9±1.1	—	—

* : Mean±SD

* : DNA band from RIR-Y8.

found between the orders of DNA fingerprinting and Z value.

Discussion

DNA fingerprint method was able to detect many loci at the same time, and was a very powerful tool for analysis of the genetic profile of population (JEFFREYS *et al.*, 1985 c ; JEFFREYS and MORTON, 1987 b). DNA fingerprinting results showed higher individual specificity (GEORGES *et al.*, 1988). Our probe DNA used was bacteriophage M13 whole DNA, which was highly informative and a powerful universal probe in almost all species. But this probe DNA could not detect the relationship between DNA fingerprint band and a quantitative trait factor like egg shell color.

Mixing DNA from individuals representing a line has been used for line identification (DUNNINGTON *et al.*, 1990). The mixed DNA can be used efficiently for linkage analysis between DNA fingerprint loci and quantitative trait loci (QTL) (PLOTSKY *et al.*, 1990). All WL-r5 females used in these studies laid brownish tinted color eggs, but we could not recognize the relationship between DNA fingerprint bands and egg shell color. Other probes which contained repetitive minisatellite sequences might work effectively for the purpose.

The BS values between WL-r5 and WL-G DNA digested with *Hae*III and *Hin*fl were 0.61 and 0.48, respectively. These values may reflect that WL-r5 shares with a half or more similar genomic DNA constitution with that of WL-G. Moreover, this result may indicate that WL-r5 chickens are not hybrid of WL-G and RIR-Y8, because the BS values between W-r5 and RIR-Y8 calculated were very low (0.26 in *Hea*III and 0.15 in *Hin*fl). Similar value between WL-G and RIR-Y8 (0.24 in *Hae*III and 0.19 in

*Hinf*I) was obtained.

The common band was detected in mixed DNA digested with *Hinf*I from WL-r5 and RIR-Y8. This common band was not detected in the WL-G DNA fingerprint. It was suggested that this common band was derived from the donor RIR-Y8 sperm DNA from the first IST chicken generation, although we could not establish that this band is relating to the egg shell color gene(s).

The primer of PCR reaction used in this study was used for the analysis of human individuality based on apolipoprotein B (Apo B) 3' hypervariable region (BOERWINKLE *et al.*, 1989). The amplification of human genomic DNA with this human specific primer could produce Apo B 3' region in large quantities without nonspecific bands. The reaction conditions used in our study were the same as those used for human DNA analysis. This primer amplified the chicken genome DNA and some bands were detected. We thought that these bands obtained were not related to Apo B 3' hypervariable region, but might reflect some unknown microsatellite repetitive sequences.

The common bands between WL-r5 and RIR-Y8 were detected in DNA fingerprint using mixed DNA digested with *Hinf*I and in PCR method. From these results, it was thought that WL-r5 chickens had, for the most part, the same genome as the WL-G genome, and had a few part of the RIR-Y8 genome component(s). This RIR-Y8 genome component(s) could be transferred from the genetically transformed chicken generations through the irradiated donor sperm.

If the genetically transformed chicken males which have donor specific DNA band related to the brown egg shell color were crossed to the females which lay brownish shell color eggs, the next generation females might lay brown color eggs more than mother females do. The egg shell color would be determined by a number of genes (McDONALD 1959, HUNTON 1962). HURST (1905), PUNNETT and BAILEY (1920), BENJAMIN (1920) and FARNSWORTH and NORDSKOG (1955) indicated that the inheritance of egg shell pigmentation depended on a large number of genes, each contributing a small amount to the trait. GOWE *et al.* (1965) suggested that some major genes causing the segregation of shell color seen in brown egg flocks probably had dominance effects based on the higher variance associated with dams as compared with sires. WEI *et al.* (1992) gives rise to a hypothesis of major genes contributing to the trait of tinted egg shell colors, and have confirmed that other alleles at the major loci might control brown egg shell colors.

We thought that all egg shell color genes were not transmitted to the individuals. In our previous study the transferred egg shell color genes from irradiated sperm were stable for at least five successive generations (YAMAMOTO and TOMITA 1992). Further studies are necessary to confirm the expression of transferred genes other than the egg shell color genes.

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遺伝的形質転換ニワトリのミニおよび マイクロサテライト DNA による解析

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ガンマー線を照射したドナー精子と非照射レシピエント正常精子により作出した遺伝的形質転換ニワトリの第5世代におけるDNAを解析した。ミニサテライトDNAの分析にはフィンガープリント法を, またマイクロサテライトDNAの分析には遺伝子増幅(PCR)法をそれぞれ用いた。供試鶏は遺伝的形質転換系WL-r5, レシピエント系WL-G, およびドナー系RIR-Y8の各雄雌である。本実験で用いた条件下では卵殻色と個体毎のDNAフィンガープリントバンドとの間に相関関係を認めることはできなかった。各系統毎に雄雌別のDNAを混合し, 系統の特徴を反映したDNAフィンガープリント像を作成した。この結果を計算式に当てはめ, 遺伝的相似性(BS値)を求めた。WL-r5とWL-Gとの比較におけるBS値は, 0.61 (*Hae*III) および0.48 (*Hin*II)であった。このBS値より推定すると, WL-rはWL-Gとの間に約半分あるいはそれ以上の相似的DNAを持つということが示唆される。WL-r5とRIR-Y8, またはWL-GとRIR-Y8の比較で得られたBS値は低く, 相似性は非常に少ないと考えられる。雄雌別の混合DNAにより作出したDNAフィンガープリントから, WL-r5とRIR-Y8に共通で, かつWL-Gには存在しないバンドが, *Hin*IIで消化したDNAにおいて1本確認された。

また, WL-r5とRIR-Y8の個体毎のDNAフィンガープリントでは, このバンドをもつものが雄で2個体, 雌で6個体に確認された。混合DNAの解析では, 系統を特徴的に示すようなバンドが見られた。PCR法による解析では, WL-r5とRIR-Y8に共通で, かつWL-Gに存在しないバンドが確認された。WL-r5雌が180日齢に産んだ卵の卵殻色の3成分(L値: 明るさ, a値: 赤色, b値: 黄色)を測定したところ, 着色の程度が高いものから低いものまで連続的な結果が得られた。3成分の平均値はWL-GとRIR-Y8のほぼ中間の値を示した。以上の結果を総合的に判断すると, WL-r5のゲノムはWL-Gと多くの相似性をもち, わずかではあるがRIR-Y8のDNAとも類似する点をもつということが推察された。RIR-Y8との相似性は, 遺伝的形質転換第1世代(WL-r1)において獲得したドナー精子由来のDNAがWL-r5まで安定的に伝達されてきた結果であろうと考えられた。

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キーワード: 遺伝的形質転換ニワトリ, ミニサテライトDNA, マイクロサテライトDNA, PCR, DNAフィンガープリント