Identification of Sex-Specific DNA Sequences in the Chicken

K. D. Song, Y. S. Shin[1] and Jae Y. Han[2]

College of Agriculture and Life Sciences, Seoul National University

Suweon 441-744, Korea

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닭의 성특이적 DNA 분리

송기덕 · 신영수[1] · 한재용[2]

서울대학교 농업생명과학대학

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적 요

닭에서 적절한 성감별 방법을 개발하고 닭의 성분화 기작의 기초자료를 얻기 위하여 배아의 섬유아세포의 염색체를 분석하고, W 염색체 특이적인 반복염기서열과 50~60%의 유사성을 보이는 random primer로 PCR 증폭을 실시하여 성을 판별하는 방법이 이용되었으며, 닭에서 성분화에 관련된 유전자를 분리하기 위해 W 염색체 특이적인 반복염기서열을 클로닝하였고, PCR을 이용하여 ZFY와 SRY 염기서열을 증폭하였다. 닭의 배아섬유세포의 염색체 분석 결과 Z 염색체와 W 염색체를 구분함으로써 배아의 성을 직접적으로 판별하는 것이 가능하였으며, 암탉의 DNA를 Xho I와 Eco RI로 절단하여 생성되는 band를 이용하여 성을 판별하는 것이 가능하였다. Xho I와 Eco RI family를 클로닝하고, colony hybridization을 통해 Xho I과 염기서열이 유사한 80~100개의 clone을 동정하여, 이들 두 그룹간 DNA homology는 매우 유사하였다. 150개의 random primer 중 W 염색체 특이적인 반복 염기서열과 유사성을 보이는 primer 7개를 screening하였으며, 이 중 3개의 primer는 닭에서 자성과 웅성간의 차이를 나타내었다. 닭에서 성분화에 관련된 유전자를 동정하기 위하여 포유류의 ZFY와 SRY 유전자의 PCR 증폭을 실시하였다. ZFY를 증폭한 결과, 자성과 웅성간의 차이를 발견할 수 없었으며, 이는 닭에서 ZFY는 상염색체 또는 Z 염색체에 존재함을 시사한다. SRY의 증폭에서는 성간의 차이가 확인되었으나, 이 유전자가 Z 염색체에 존재하는지 W 염색체에 존재하는지 혹은 상염색체 존재하는지 여부는 연구가 필요하리라 사료된다.

(색인 : 성 감별, Z 염색체, W 염색체, RAPD, PCR, ZFY, SRY)

Ⅰ. INTRODUCTION

Predetermination of embryonic sex in mammalian, especially in cattle, pig, and sheep is meaningful in animal breeding strategies due to its economical benefits. The mechanism by which the sex of an individual is determined has

been a theme of scientific speculation. Jacobs and Strong (1959) reported that Y chromosome in mammals played critical roles in sex determination, and the embryos having Y chromosome developed as males whereas the embryos lacking Y chromosome as females.

With the advance of recombinant DNA techniques, it has been possible to isolate the Y-specific repetitive sequences (Ellis et al., 1989) and the coding sequences, such as ZFY (Page et al., 1987), SRY (Berta et al., 1990). The ZFY (zinc finger Y) has been identified in a small portion of human Y chromosome and it encodes a protein with multiple finger domain, designated as zinc finger Y (ZFY). This protein had a similar amino acid sequence to transcription factor IIIA in *Xenopus*, suggesting its function as a trans-acting transcription factor regulating the expression of genes which may be involved in sex differentiation. Very similar sequences were identified and cloned in marsupials (Sinclair et al., 1988), mice (Mardon et al., 1989 ; Nagamine et al., 1989), sheep, goat and cattle (Pollevick et al., 1992), and amphibian (Elizabeth et al., 1992). However, the role of ZFY in sex determination is not still clear.

Sinclair et al. (1990) reported another putative sex determining gene in Y chromosome, and termed SRY (sex determining region of Y). They mapped this gene to the boundary between pseudoautosomal region in Y chromosome which shows high homology to X chromosome counterpart. With seven of 50 probes from overlapping clones, they screened the region of Y chromosome and one of them hybridized most strongly to Y-specific fragments in human, murine and bovine genomic DNA. The nucleotide sequences of this probe, pY53.3, showed that one of the open reading frames has high similarity to both a portion of the Mc protein of the

yeast *S. pombe* and a conserved motif in HMG1 and HMG2. The homologous sequences of SRY were reported in other species, such as mice (Gubbay et al., 1990), marsupials (Foster et al., 1992), sheep, cattle and goats (Kageyama et al., 1992 ; Payen and Continot, 1993). The sequence homology of SRY-conserved motif in mammals shows that the motif is highly conserved and it may play an important role in sex differentiation. Koopman et al. (1990) reported that transgenic female mouse with SRY gene had male specific characteristics, suggesting that SRY gene is sex determining gene in mammals.

On the contrary to mammal, in aves, the study about the mechanism of sex determination was in infancy. The Z chromosome of chicken was identified as the 5th large chromosome by Hutt et al. (1949) and the W chromsme was demonstrated by Owen (1965) in female chicken. Therefore, avian species are homogametic male (ZZ) and heterogametic female (ZW). Only a few genes were mapped in chicken W chromosome. Bloom et al. (1974) reported that there are W-linked histocompatibility locus in the chicken, but the molecular nature of this locus is unknown. The repetitive DNA sequences which are W chromosome-specific were cloned and sequenced by Tone et al. (1984) and Saitoh et al. (1991). Tone et al. (1982, 1984) digested female genomic DNA with restriction enzyme Xho I and cloned the 1.1.kb and 0.7kb band. The Xho I family is estimated to account for 46% of the chicken W chromosome. Saitoh et al. (1991) also reported another W chromosome-specific repetitive sequences, Eco RI family in the W chromosome, in which the fragment of about 1.2kb was produced by Eco RI digestion. The two families had a common internal repeat structure, although they are distinguishable by hybridization. The function of these families and

whether these families are linked to the genes expressed in W chromosme remain to be characterized.

The chicken W chromosome is known to be heterochromatin and consists of highly repetitive sequences with less functional genes. It is important to analyze the genetic characteristics and the mechanisms of chicken sex differentiation and isolate the putative genes which may play a role in chicken sex differentiation. The objectives of this study were to isolate the W chromosome-specific clone and develop the PCR sexing techniques in order to provide the information of chicken sex differentiation mechanisms and apply PCR sexing technique to other species. The possibility of sex determination using random primers with sequence homology to W chromosome-specific repetitive sequences was described and ZFY and SRY related sequences were identified on the chicken genome by amplification of mammalian conserved sequences.

II. MATERIALS AND METHODS

1. Genomic DNA preparation

Male and female chicken genomic DNA were prepared from the White Leghorn blood cells. One milliliter of peripheral blood was drawn from the wing vein of inbred White Leghorn chickens into a sterile syringe containing sodium heparin. After washing with equal volume of 1X SSC, 50 μl of blood cells was suspended in 1 ml of high TE (100 Mm Tris-Cl pH 8.0, 40 mM EDTA pH 8.0), and lysed in 1 ml of lysis buffer (100 mM Tris-Cl pH 8.0, 40 mM EDTA pH 8.0, 0.2% SDS). The lysates were extracted several times with phenol : chloroform : isoamylalcohol (25:24:1) to isolate high molecular weight genomic DNA as described by Sambrook et al.

(1989). A spectrophotometer was implemented for quantifying the amount of DNA.

2. Chromosome observation

Chicken embryo fibroblasts (CEF) were prepared from the skin of 4 to 6 day incubated embryos for sex identification. Cell dissociation was accomplished with 1X trypsin-EDTA in phosphate-buffered saline (Ca^{2+} and Mg^{2+} free) for 5 min. Cells were mechanically dispersed by repeated aspiration with a Pasteur pippet. CEF cells were grown in a medium consisting of 50% Dulbecco's modified Eagle's medium (DMEM) and 50% Ham's nutrient mixture F-12 (Sigma), supplemented with 10% calf serum (or chicken serum), 1% antibiotic-antimycotic (Sigma) and 1% L-glutamine (Sigma). The sodium bicarbonate level of this medium was 2.2 g /liter. Cultures were maintained at 41℃ in a humidified incubator with 5% CO_2. At 71 hr of incubation, 0.05% colcemid was added to each culture flask and the cultures were incubated for 1 hr in order to maximize the number of metaphase chromosomes. Cells were immediately centrifuged at 1,000 rpm for 10 min, resuspended in 0.067 M KCl and incubated at 37℃ for 10 min for hypotonic treatment. Cells were then centrifuged at 1,000 rpm for 10 min and resuspended in fresh, cold methanol:acetic acid (3:1) fixative. The fixative was added drop by drop while carefully aspirating the cell suspension. After the cells were allowed to fix for 20 to 30 min in the first fixative, the fixative was changed 2 to 3 times. Chromosome preparations were made by resuspending the cells with the proper amount of fixative to give an optimum cell density and dropping the cells onto a slide from an 75 to 90 cm height.

3. DNA analysis

DNA samples of male and female chickens

were digested with either restriction endonuclease Xho I or Eco RI (20units/8 μg DNA, Kosco Co.) overnight at 37℃ in enzyme buffer (6 mM Tris-Cl pH 8.0, 6 mM MgCl₂, 150 mM NaCl, 1 mM DTT for Xho I ; 50 mM Tris-Cl pH 8.0, 10 mM MgCl₂, 100 mM NaCl for Eco RI). The cleaved DNA was subjected to submarine agarose gel electrophoresis on a 1% agarose gel containing 0.5 μg/ml ethidium bromide for 18 hr at 35 Volts in 1X TAE buffer (0.04 M Tris-acetate, 0.01 M EDTA). Complete digestion of DNA samples was confirmed by repeated analysis.

4. Cloning of W chromosome-specific DNA sequences

Cloning procedure for W chromosome-specific DNA sequences was followed from the methods described by Sambrook et al. (1989). The 1.2kb band of Eco RI family was eluted from the low melting agarose gel and ligated into Eco RI digested pUC19 plasmid vector. The pUC19 plasmid was isolated by miniprep, digested with Eco RI (10 μg / 50 U) and dephosphorylated with calf intestinal alkaline phosphatase (1 U, Kosco Co.) in CIP buffer (1 m. ZnCl₂, 1 mM MgCl₂, 1 mM Tris-Cl pH 8.3). Ligation of 1.2kb fragement into linearized plasmid vector was performed with T4 DNA ligase (0.1 Weiss unit) in a ligation mixture containing T4 DNA ligase buffer (20 mM Tris-Cl pH 7.6, 5 mM MgCl₂, 5 mM dithiothreitol, 50 μg/ml bovine serum albumin) and 0.5 mM ATP.

The recombinant molecules were transformed into *E. coli* strain JM109 by calcium chloride (Sambrook et al., 1989). The colony containing recombinant was detected with X-gal/IPTG (20 mg/ml X-gal : 200 mg/ml IPTG, Jersey Lab Supply) treatment by identification of white color. To perform colony hybridization,

0.7kb band produced by Xho I digestion of genomic DNA was eluted from the low melting agarose gel and labelled with digoxigenin-11-dUTP labelling kit as described by supplier's manual (Boehringer-Mannhneim Co.). Hybridization and washing were performed at low stringency (washing solution: 5X SSC, blocking reagent, 0.1% N-lauroylsarcosine, 0.02% SDS, washing solution : 2X SSC containing 0.1% SDS or 0.1X SSC containing 0.1% SDS). After washing, the signal was detected by incubation with anti-digoxigenin Fab fragment conjugated to alkaline phosphatase and then with colorimetric alkaline phosphatase substrate.

5. DNA amplification

For RAPD (random amplified polymorphic DNAs) analysis, the male and female genomic DNA (200 ng/μl) were added to PCR mixture consisting of 10X reaction buffer (100 mM Tris-Cl pH 8.3, 50 mM KCl, 20 mM MgCl₂, 1% trition X-100), dNTP mixture (200 μM each), 0.5 unit of *Taq* DNA polymerase (Korea Biotech. Inc.) and 0.2 μM random primer (purchased from University of British Columbia, Canada). The sequences of random primer used in this study are shown in Table 1. Twenty five

Table 1. Sequences of random primers used in this study.

Primer	Sequences
#1	5'-TTC CCC GCC C-3'
#2	5'-GCT GGT ACC C-3'
#3	5'-ACT TCC TCC A-3'
#4	5'-GCG AAC CTC C-3'
#5	5'-ATG GCA AAG C-3'
#6	5'-TGG ACC ACC C-3'
#7	5'-GAG ATC CCT C-3'

The primers show the 50~60% homology to the repetitive sequences of Xho I or Eco RI family

Table 2. Lists of primer sequences for ZFY and SRY amplification

Primer	Sequences	Reference
ZFY1	5'-ATAATCACATGGAGAGCCACAAGCT-3'	Schneider-Gädicke et al.(1989)
ZFY2	5'-CATTATGTGCTGGTTCTTTTCTG-3'	Schneider-Gädickee et al.(1989)
SRY1	5'-AAGCGACCCATGAACGCATT-3'	Sinclair et al.(1990)
SRY2	5'-GTATTTCTCTCTGTGCATGG-3'	Sinclair et al.(1990)

microliters of total volume was used and same volume of mineral oil was overlaid. Amplification was conducted in a Perkin Elmer Cetus DNA thermal cycler. Denaturation was performed at 94℃ for 5 min and followed by 50 cycles; denaturation for 1 min at 94℃, annealing for 1 min at 39~50℃, extension for 2 min at 72℃, final extension for 5 min at 72℃, the termination of enzyme reaction and storage at 4℃.

To amplify the ZFY and SRY-related sequences in the chicken, upward and downward primers were designed (Table 2). The ZFY primers were synthesized according to the published sequences in human and cattle (Schneider-Gädicke et al., 1989; Aasen and Medrano, 1990). The SRY primers were made to amplify the HMG-box motif (Sinclair et al., 1990). Amplifications for ZFY and SRY sequences were performed with 40 cycles consisting of denaturation at 94℃ for 1 min, annealing at 55 or 60℃ for 1 min, extension at 72℃ for 1 min. After PCR amplification, the amplification products were analyzed by electrophoresis in 2% agarose gel and detected by ethidium bromide staining.

Ⅲ. RESULTS AND DISCUSSION

1. Chromosome observation

The results of chromosome preparation from embryonic fibroblast are shown in Fig. 1. Total 78 chromosomes were observed in methaphase. Embryo having ZW chromosme was considered as a female embryo (Fig. 1A), and having ZZ chromosome was a male (Fig. 1B). The arrows
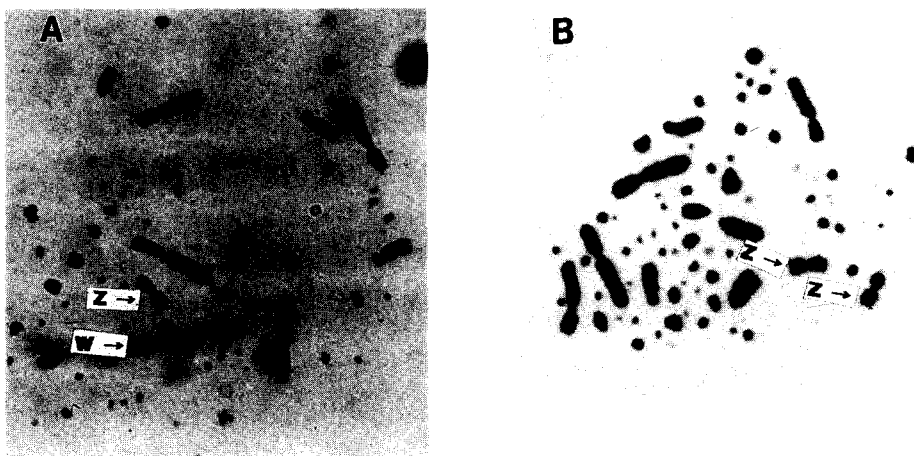

Fig. 1. Chromosome analysis of chicken embryonic fibroblast. Arrows indicate the sex chromosome. A. Female, B. Male.

indicated in Fig. 1A and B are the Z and W chromosomes, respectively. In spite of the availability of chromosome preparation in chicken embryo, its practical use of sexing is limited because of the difficulties in preparing the samples without death and need of a large number of cells, compared with PCR sexing techniques.

2. Sex determination by the genomic DNA digestion and cloning of W chromosome-specific fragment

Fig. 2 shows that DNA band differences between sexes are detected on the agarose gel by Eco RI restriction enzyme digestion. In male there is no band, but there is one 1.2 kb band in female. With Xho I restriction enzyme digestion, two bands (1.1 kb, 0.7 kb) were detected
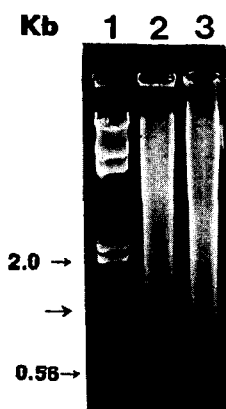
in female genomic DNA (Data not shown), suggesting that sex identifcation of chicken can be performed by simple methods of DNA digestion. Fig. 3 shows the cloned 1.2 kb Eco RI fragment. The recombinant clones which contained the 1.2 kb Eco RI frgament were about 60% of the total 300 clones. Fig. 4 shows the results of colony hybridization of Eco RI 1.2 kb band library with the labelled probe of 0.7 kb Xho I fragment. The number of hybridized colonies was about 80~100, implying that 0.7 kb Xho I family is very homologous with 1.2 kb Eco RI family in chicken W chromosome-specific repetitive sequences. These hybridized clones might be used for the synthesis of W chromosome-specific primer and the insert fragment can be used as probes to isolate the large fragments of W chromosome which are digested with rare cutter restriction enzyme.

The cloning and sequencing of these two W chromosome-specific repetitive DNA sequences families make it possible to determine the sex of chicken embryo by simple method. After incubation of fertilized eggs positioned on their sides
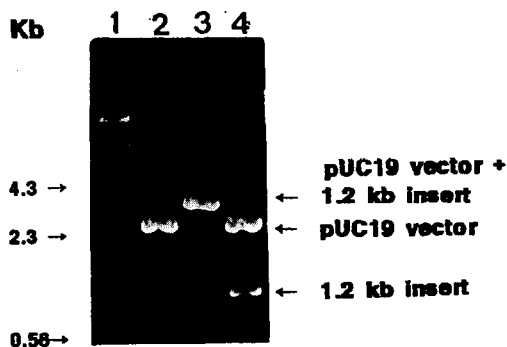

Fig. 2. Restriction endonuclease digestion of male and female genomic DNA. A faint band (1.2kb) indicated by arrow is shown in female genomic DNA (lane 2), not in male genomic DNA (lane 3). Lane 1 : Size marker (Lambda-Hind III digested), Lane 2 : Female genomic DNA digested with Eco RI, Lane 3 : Male genomic DNA digested with Eco RI.

Fig. 3. Cloning of 1.2 kb female specific band. Lane 1 : Size marker (Lambda-HindIII digested), Lane 2 : pUC 19 plasmid vector, Lane 3 : Recombinant (pUC19 + 1.2 kb Eco RI fragment), Lane 4 : Vector pUC19 and 1.2 kb insert digested with Eco RI.

Fig. 4. Colony hybridization of 1.2 kb Eco RI library with digoxigenin-11-dUTP labelled 0.7 kb Xho I fragment.

for 2 or 3 days, a square window can be cut in the site of the embryo (top of the egg) for removal of several cells from the extraembryonic membrane to perform DNA analysis by PCR. Because the nucleotide sequences of two families are available (Mizuno et al.,1993), it is possible to perform the PCR for the sex determination. Or the extracted DNA can be hybridized with Xho I clone or Eco RI clone. Xho I and Eco RI clones may be useful in the identification of functional genes on the W chromosome. These sequences may be used as molecular landmark, STS (sequence tagged site) for W chromosome.

3. Random amplified polymorphic DNAs (RAPD) to find out W chromosome-specific sequences

To develop the method for sexing and obtain the basic information for sex related genes in the chicken, we attempted to apply the RAPD techniques by arbitrary primed PCR. RAPD was originally deviced to maximize the detection of DNA polymorphism (William et al., 1990). We hypothesized that some random primers may anneal to the W chromosome, and amplification of a portion of W chromosome is feasible. To prove this hypothesis, we initially screened the 150 random primers to select the primer suitable to our study. Seven primers were selected and used to this study. The sequences of primers shown in Table 1 are about 50~60% homologous to the repeating DNA unit of Xho I or Eco RI family (Xho I repeating sequences: GAAAA-TACCACNTTTTCTCCC; Eco RI repeating sequences: GAAAATACCNTTTTCTCCC). The results of RAPD are shown in Fig. 5. The primers (#1, #2 and #4) was useful for the identification of differences between sexes as indicated by arrows in Fig. 5. The PCR products from female chicken genomic DNA showed more band than male genomic DNA, suggesting that W chromosome-specific DNA sequences might be amplified. These female specific PCR products can be used for mapping and cloning of W chromosome-specific sequences and need to be specified for further study of sexing mechanism. The RAPD was performed at different an-
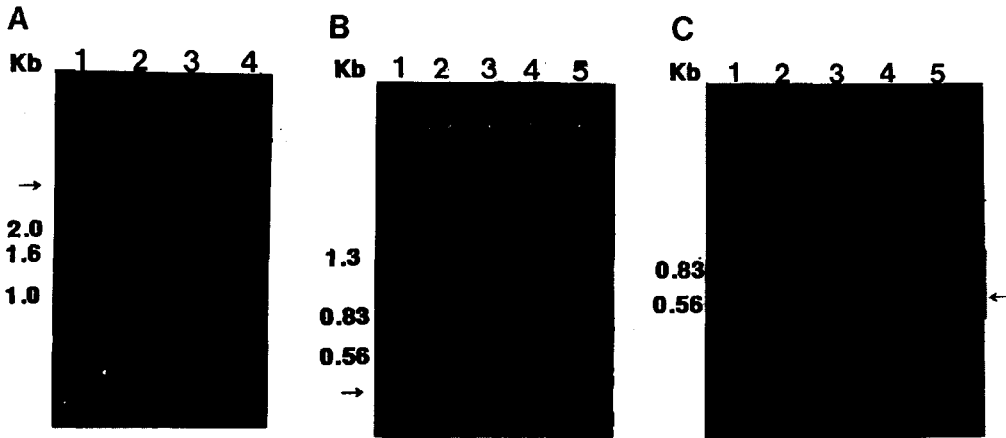
Fig. 5. Profiles of random amplified polymorphic DNAs(RAPD) in the chicken genomic DNA. DNA amplification was performed with 50 cycles at the following conditions : denaturation for 1 min at 94℃, annealing for 1 min at 45℃, extension for 2 min at 72℃. DNA bands were detected by ethidium bromide staining after electrophoresis in 2% agarose gel. The PCR products expected for sexing are indicated by arrows.

A. Primer #1, Lane 1 : Size marker (1 kb DNA ladder), Lane 2 : Chicken female, Lane 3 and 4 : Chicken male. B. Primer #2, Lane 1 : Size marker (Lambda-Hind III and Eco RI digested), Lane 2 and 3 : Chicken female, Lane 4 and 5 : Chicken male. C. Primer #4, Lane 1 : Size marker (Lambda-Hind III and Eco RI digested), Lane 2 and 3 : Chicken female, Lane 4 and 5 : Chicken male.

nealing condition (annealing temperature ; 39℃, 45℃, 50℃). When annealing temperature was raised to 45℃ to minimize the non-specific binding, band pattern was not changed from the condition of 39℃ annealing temperature. Some female specific band disappeared(data not shown) in annealing condition at 50℃, due to the high stringency condition.

4. Identification of ZFY and SRY related sequences in the chicken

ZFY and SRY are mapped on Y chromosome in mammals (Page et al., 1987; Berta et al., 1990). However, sex control or related genes in the chicken are not identified yet and only a few genes were mapped on chicken Z and W chromosomes. Page et al. (1987) reported that the clone pDP1007 derived from human Y chromosome was hybridized with male and female chicken genomic DNA, implying that a part of chicken genomic DNA has homologous region with mammalian genome. We performed PCR on the assumption that some X or Y-linked genes in mammals may be located on Z or W chromosome in the chicken. The primers covering the conserved portion of human X and Y chromosome were made and used for the amplification of ZFY related sequences in the chicken. ZFX or ZFY related sequences are amplified with the chicken genomic DNA, but there was no different profile between sexes (Data not shown). This result leads to the conclusion that the ZFX or ZFY related sequences may exist in the chicken and locate on autosome or Z chromosome possibly. To clarify the chromo-

somal location of ZFX or ZFY related sequences in the chicken, the pedigree analysis and RFLP analysis should be followed. The SRY related sequences in the chicken are shown in Fig. 6. As genomic DNA content increases, some bands disappear or appear. The difference of bands between sexes was clearly observed in 800 ng /25 μl of genomic DNA. The
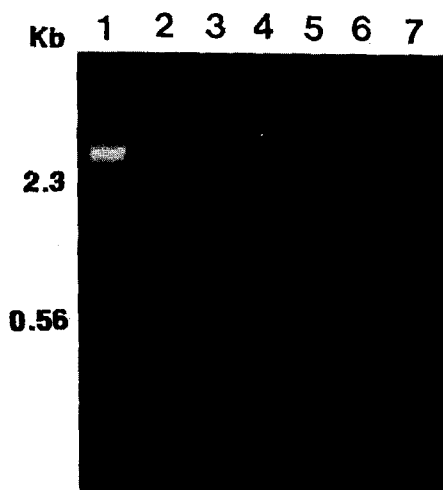

Fig. 6. Identification of SRY-related sequences in the chicken by PCR with different DNA amount. PCR amplification was performed in the PCR mixture containing 10X reaction buffer (100 mM Tris-Cl pH 8.3, 50 mM KCl, 20 mM MgCl₂, 1% triton X-100), dNTP mixture (200 μM each), 1 unit of *Taq* DNA polymerase and 0.5 μM of upward and downward primers. Lane 1 : Size marker (Lambda-Hind III and Eco RI digested), Lane 2 : Chicken male (800 ng genomic DNA), Lane 3 : Chicken male (400 ng genomic DNA), Lane 4 : Chicken male (200 ng genomic DNA), Lane 5 : Chicken female (800 ng genomic DNA), Lane 6 : Chicken female (400 ng genomic DNA), Lane 7 : Chicken female (200 ng genomic DNA).

reason why many bands appear is that the primer used in this study was designed to cover the HMG-conserved motif of SRY gene and the primer of 21 mer was not long enough to specify SRY-related sequences. Some bands may be HMG-related sequences, but which band is true SRY related sequence in the chicken is unclear in this data. This kind of difficulties was described by Kageyama et al. (1992) and they tried to raise stringency by elongating the primers to 31 mer and making primers corresponding to more inner sequence than HMG-conserved box. These strategies might be useful in the amplification of SRY-related sequences in the chicken. In conclusion, ZFY and SRY sequence was amplified successfully in the chicken genome, implying that chicken genome might have the sex-related conserved sequences similar to mammalian ones. To characterize the true sex-related sequences, further study such as Southern hybridization, cloning and sequencing are necessary. Application of this techniques to the study of gene expression concerning sex control gene during sex differentiation of chicken might be very powerful tool.

IV. ABSTRACT

This study was performed to find out the reasonable sexing methods in the chicken, obtain the basic information for the mechanisms related to chicken sexual differentiation and identify the genes which known to involved in chicken sex differentiation. The chromosome analysis of chicken embryonic fibroblast was a simple method to determine sex of chicken by means of Z and W chromosome identification. The bands of female chicken genomic DNA

digested with Xho I and Eco RI restriction endonuclease showed to be useful in direct sex determination and these repetitive sequences of Xho I and Eco RI families were proposed to be very homologous in their sequences by colony hybridization analysis. Seven of 150 random primers were selected to amplify the W chromosome-specific band by using arbitrary primed PCR and three of them were useful to identify the sex of chicken. To identify the sex differentiation genes in the chicken, PCR for the amplification of ZFY and SRY sequences was performed. ZFY and SRY sequences were amplified successfully in the chicken genome, implying that chicken genome might have the sex-related conserved sequences similar to mammalian ones. The PCR products of ZFY amplification were the same in both sexes, suggesting that these sequences may be located on autosome or Z chromosome. The profile of PCR amplification for SRY sequences showed variation between sexes, but this result was not enough to specify whether the SRY gene in chicken is on the autosome or sex chromosome.

(Key words : chicken sexing, Z and W chromosome, RAPD, RCR, ZFY, SRY)

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