Resolution of High Molecular DNAs in W Chromosome of the Domestic Fowl by Pulsed Field Gel Electrophoresis

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The W chromosomal DNA fragments of the domestic chicken, *Gallus gallus domesticus*, and Japanese quail, *Coturnix coturnix japonica*, were separated as bands by pulsed field gel electrophoresis after digestion with restriction endonucleases. These fragments were detected with 14 kinds of restriction endonucleases out of the 20 kinds in the chicken and only 1 kind of restriction endonucleases out of 16 kinds in the quail. In the chicken, many of these fragments were molecules greater than 2 megabasepairs (Mb) in size and hybridized with the W chromosome-specific 0.7 Kb *Xho*I repetitive DNA sequences of the chicken. However, the W chromosomal DNA fragments which did not show hybridization with the 0.7 Kb *Xho*I repeating unit were detected as 3 bands with *Dra*I digestion and as 4 bands with *Eco*RI digestion up to a size less than 2 Mb in the chicken. The results may suggest that there could be some other repeating units than has been reported before.

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Key words: domestic fowl, W chromosome, repetitive DNA sequence, pulsed field gel electrophoresis

Introduction

Recent technical developments in pulsed field gel electrophoresis have led to separation of DNA fragments greater than 10 mega-basepairs (Mb). The local distribution of sequence variation within large arrays of tandemly repeated α -satellite DNA from human chromosome 17 (WARBURTON and WILLARD, 1990), the physical mapping of human chromosome 21 (GARDINER *et al.*, 1988), and the estimates of the size of the *Giardia lamblia* genome (FAN *et al.*, 1991) have been performed by using pulsed field gel electrophoresis.

The W chromosome of the birds is inherited only by female. In the chicken, W chromosome is different from other chromosome having about 70~90% of the W chromosomal DNA occupied by the W chromosome-specific repetitive DNA sequences (SAITOH *et al.*, 1991). These sequences seem to constitute the heterochromatin region in chromosomes and have been also detected in turkey, pheasant and Bobwhite quail (SAITOH *et al.*, 1989). However, it is not clear how large the arrays to be composed by these repetitive sequences.

In this study, the W chromosomal DNA fragments were separated as specific and repeatable bands by pulsed field gel electrophoresis.

Materials and Methods

Animals

Two lines of Japanese Game chicken (*Gallus gallus domesticus*) and Japanese quail (*Coturnix coturnix japonica*) were used in this study. Japanese Game chicken used were 4 males and 6 females, and Japanese quail used were 3 males and 3 females bred from a pair of parents.

Preparation of DNA for pulsed field gel electrophoresis

Extremely high molecular weight DNAs were prepared from red blood cells by applying the agarose plug protocol as described by MANIATIS *et al.* (1989). Nuclei were separated from red blood cells lysed with 1% Triton X-100 solution (0.32 M Sucrose, 10 mM Tris-HCl, pH 7.6, 5 mM MgCl₂ and 1% Triton X-100) (CIULLA *et al.*, 1988). Approximately 1.75×10^8 nuclei/ml of agarose plug was made of 0.5% low-temperature-agarose (Agarose GB, Nippon Gene Co.) in L buffer (0.1 M EDTA, pH 8.0, 10 mM Tris-HCl, pH 7.6, 20 mM NaCl). A plug was incubated twice in 16 volumes of L buffer containing 1 mg/ml proteinase K and 1% Na-N-lauroyl sarcosine for 24 h at 50°C. Then the plug was treated twice in 16 volumes of TE (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, pH 8.0) containing 40 µg/ml PMSF (phenylmethylsulfonyl fluoride) for 1 h at 50°C. After the agarose plug was cut into 10 µl of gel blocks (5 × 2 × 1 mm) containing 4–5 µg DNA with a sterilized spatula, the block was digested with 40 units of an appropriate restriction endonuclease recognizing 6 or 8 basepairs for 16 h at the optimal temperature in a total volume of 50 µl.

Pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) was performed in 1% or 0.8% agarose gel using the CHEF-DRII system (Bio-Rad) in 0.5 × TBE (25 mM Tris, 24 mM boric acid, 0.5 mM EDTA) at 14°C. Electrophoretic parameters were 60 sec pulse time at 200 V for 15 h followed by 90 sec pulse time at 200 V for 9 h. DNA size markers used were yeast chromosomal DNAs (*Saccharomyces cerevisiae*, Bio-Rad).

Detection of the bands from W chromosomal DNA fragments

After PFGE, the gel was stained with 0.5 µg/ml ethidium bromide for 30 min and washed in distilled water overnight. The bands detected in females and not detected in males with ultraviolet radiation were regarded as the W chromosomal DNA fragments.

Southern blot hybridization

After ethidium bromide staining, the gel was treated with 0.25 N HCl for 30 min, incubated in 0.4 N NaOH and 1 M NaCl for 1 h, and treated with 1 M Tris-HCl (pH 7.4) and 1.5 M NaCl for 1 h. Then DNA fragments were transferred to the Hybond-N+ filter (Amersham) in 10 × SSC (1.5 M NaCl and 0.15 M Na citrate) for 48 h by capillary Southern blotting (SOUTHERN, 1975). Prehybridization and hybridization were carried out using the ECL (enhanced chemiluminescence) gene detection system (Amersham) without radioisotope. After hybridization, the DNA filter was washed twice in primary wash buffer (6 M urea, 0.4% SDS and 0.5 × SSC) for 20 min at 42°C, and rinsed twice in 2 × SSC for 5 min at room temperature. The DNA filter was soaked in the detection

solutions (ECL system, Amersham) for precisely 1 min at room temperature. Then, as quickly as possible, a sheet of Hyperfilm-ECL (Amersham) was exposed on the blot for 15 min at room temperature. The hybridization signal patterns were detected by fluorometry. The probe used in this study was W chromosome-specific 0.7 Kb *Xho*I repeating unit (a 0.7 Kb insert from pUGD0600, TONE *et al.*, 1984).

Results

Separation of high molecular W chromosomal DNA fragments by pulsed field gel electrophoresis

In the chicken and quail light/dark banding patterns were observed very clearly in ethidium bromide staining, as shown in Fig. 1. The patterns appeared in the wide range from a few hundred kilo-basepairs to a few mega-basepairs (Mb). The bands from the W chromosomal DNA fragments in chickens were detected only in females and not in males. The female specific bands were detected in 14 kinds of restriction endonucleases, *Bam*HI, *Ban*III, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, *Sma*I, *Xba*I and *Xho*I. This result was summarized in Table 1. Many of the detected bands were observed in the region greater than 2.2 Mb. Eight bands and 4 bands were recognized in digestion especially with *Dra*I and *Eco*RI, respectively, and 5 bands and

Fig. 1. Detection of the W chromosomal DNA fragments of female Japanese Game chickens of the ethidium bromide-stained PFGE patterns of *Hind*III digests on 0.8% agarose gel. Arrows show the W chromosomal DNA fragments. Lane M contains DNA size marker, chromosome-sized DNAs of *Saccharomyces cerevisiae*.

Table 1. The number of restriction fragments from W chromosome of Japanese Game chicken on staining with ethidium bromide after PFGE

Restriction endonucleases	The number of restriction fragments		Total
	>2.2 Mb*	<2.2 Mb (band sizes, Mb)	
<i>Aat</i> I	–	–	–
<i>Bam</i> HI	2 (H, L)**	1 (1.6)	3
<i>Ban</i> III	1 (H)	–	1
<i>Bgl</i> I	–	–	–
<i>Bgl</i> II	2 (H, L)	–	2
<i>Bst</i> EII	–	–	–
<i>Dra</i> I	3 (2H, L)	5 (1.5, 1.4, 1.2, 1.1, 0.95)	8
<i>Eco</i> RI	–	4 (1.5, 1.1, 1.05, 0.8)	4
<i>Eco</i> RV	2 (2L)	–	2
<i>Hind</i> III	2 (2L)	–	2
<i>Kpn</i> I	1 (L)	–	1
<i>Not</i> I	–	–	–
<i>Pst</i> I	2 (H, L)	–	2
<i>Pvu</i> II	1 (L)	–	1
<i>Sac</i> I	1 (L)	–	1
<i>Sac</i> II	–	–	–
<i>Sal</i> I	–	–	–
<i>Sma</i> I	1 (L)	–	1
<i>Xba</i> I	2 (H, L)	–	2
<i>Xho</i> I	1 (L)	–	1

* Mb = 10³ Kb

** A few bands were detected with molecules greater than 2.2 Mb in size. The one with higher molecular size is designated H, whereas the one with lower molecular size is named L. PFGE was carried out on 0.8% agarose gel for *Dra*I, *Hind*III, *Pst*I and *Pvu*II, and on 1% agarose gel for other restriction endonucleases.

4 bands in the observed bands were molecules less than 2.2 Mb in size. However, in the quail, a band from W chromosomal DNA fragments was detected only in *Dra*I digests (data not shown).

Identification of the female specific bands by hybridization with the W chromosome-specific 0.7Kb XhoI repeating unit

Hybridization patterns were detected with the W chromosome-specific 0.7 Kb *Xho*I repeating unit after the intact genomic DNAs were digested with 20 restriction endonucleases and separation by PFGE. As shown in Fig. 2, a few hybridization signals were observed in the range greater than 2.2 Mb in digestion with 16 restriction enzymes, *Bam*HI, *Ban*III, *Bgl*II, *Bst*EII, *Dra*I, *Eco*RV, *Hind*III, *Kpn*I, *Not*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, *Sma*I, *Xba*I and *Xho*I. These signals can be classified into 2 groups, one group with higher molecular size and the other with lower molecular size. However, most of signals remained only in origin in *Aat*I, *Bgl*I and *Sac*II, and smeary signals were appeared in the range from 400 Kb to 700 Kb in *Eco*RI and from 350 Kb to 750 Kb in *Pvu*II. The number and size of these signals were summarized in Table 2.

In comparison of these hybridization signals and the bands from the W

Fig. 2. Detection of the W chromosomal DNA fragments of female Japanese Game chickens by Southern blot hybridization with the W chromosome-specific 0.7 Kb *Xho*I repeating units after PFGE on 1% agarose gel.

chromosomal DNA fragments in ethidium bromide staining, the signals corresponded to all bands in digestion with 12 restriction endonucleases, *Bam*HI, *Ban*III, *Bgl*II, *Eco*RV, *Hind*III, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, *Sma*I, *Xba*I and *Xho*I. However, the signals did not correspond to 3 bands (1.5 Mb, 1.4 Mb and 0.95 Mb) in *Dra*I and to all bands (1.5 Mb, 1.1 Mb, 1.05 Mb and 0.8 Mb) in *Eco*RI (Fig. 3).

Discussion

We investigated separation of the W chromosomal DNA fragments by pulsed field gel electrophoresis in Japanese Game chicken and Japanese quail. As shown in Fig. 1, clear banding patterns were appeared in ethidium bromide staining with many restriction endonucleases recognizing 6 basepairs in the range from a few hundred kilo-basepairs to a few mega-basepairs. The bands regarded as the W chromosomal DNA fragments were separated clearly, as shown in Fig. 1, in the chicken by compar-

Table 2. The number of hybridization signals detected as bands with the W chromosome-specific 0.7 Kb *XhoI* repetitive DNA sequences

Restriction endonucleases	The number of hybridization signals		
	>2.2 Mb*	<2.2 Mb (band sizes, Mb)	Total
<i>AatI</i>	– (Ori)**	[400–560]****	–
<i>BamHI</i>	2 (H, L)***	1 (1.6)	3
<i>BanIII</i>	1 (H)	[500–800]	1
<i>BglI</i>	– (Ori)	–	–
<i>BglII</i>	2 (H, L)	2 (1.7, 1.15)	4
<i>BstEII</i>	2 (Ori, H, L)	–	2
<i>DraI</i>	3 (2H, L)	2 (1.2, 1.1)	5
<i>EcoRI</i>	–	[400–700]	–
<i>EcoRV</i>	3 (H, 2L)	2 (0.7, 0.4)	5
<i>HindIII</i>	3 (H, 2L)	6 (1.3, 0.77, 0.65, 0.6, 0.52, 0.42)	9
<i>KpnI</i>	2 (H, L)	–	2
<i>NotI</i>	2 (Ori, H, L)	–	2
<i>PstI</i>	2 (H, L)	5 (1.08, 0.95, 0.8, 0.42, 0.3)	7
<i>PvuII</i>	3 (2H, L)	[350–750]	3
<i>SacI</i>	2 (H, L)	–	2
<i>SacII</i>	– (Ori)	–	–
<i>Sall</i>	2 (H, L)	–	2
<i>SmaI</i>	2 (H, L)	–	2
<i>XbaI</i>	2 (H, L)	7 (0.77, 0.75, 0.72, 0.51, 0.46, 0.44)	9
<i>XhoI</i>	2 (2L)	–	2

* Mb=10³ Kb

** Ori : The origin detected hybridization signal.

*** A few bands were detected with molecules greater than 2.2 Mb in size. The one with higher molecular size is designated H, whereas the one with lower molecular size is named L.

**** [] : Range (Kb) of hybridization signals not detected as bands

PFGE was carried out on 0.8% agarose gel for *DraI*, *HindIII*, *PstI* and *PvuII*, and on 1% agarose gel for other restriction endonucleases.

ison with males and females. Thus, it is available for investigation of the genomic arrangement because high molecular weight DNAs can be separated as bands by PFGE.

Whereas the bands from the W chromosomal DNAs in the chicken were detected in digestion with 14 restriction endonucleases (Table 1), only a 0.9 Mb band which was not clear in ethidium bromide staining was detected in *DraI* digests in the quail. Therefore genomic arrangement of the W chromosome in the chicken seems to be quite different from that in the quail in spite of the fact that they belong to the same family, *Phasianidae*. TONE *et al.* (1984) showed that the W chromosome-specific *XhoI* family repeating units of the chicken did not hybridize with both DNAs from male and female Japanese quail. It has been shown that the Bkm satellite DNA from a female Indian banded krait (*Bungarus fasciatus*) hybridized with DNA from Japanese quail with a ratio of hybridization to the female DNA/male DNA of 2.41 (JONES and SINGH, 1982). MAYR *et al.* (1989) studied resolution of the quail karyotype and characterization of

Fig. 3. Resolution of the W chromosomal DNA fragments of female Japanese Game chickens. A : Ethidium bromide-stained PFGE patterns of *Eco*RI digests on 1% agarose gel. B : Hybridization patterns with the W chromosome-specific 0.7 Kb *Xho*I repeating units. Open arrows show the W chromosomal DNA fragments detected on A and not detected on B. Lane M contains DNA size marker, chromosome-sized DNAs of *Saccharomyces cerevisiae*.

microchromosomes by counterstain-enhanced fluorescence and found that W chromosome and microchromosomes showed positive fluorescence with chromomycin A₃. Therefore the W chromosomal DNA sequences of Japanese quail are different from that of the chicken by the accumulating evidences discussed so far.

As shown in Fig. 2 and Table 2, a few hybridization signals which could be classified into 2 groups were detected with molecules greater than 2.2Mb in digestion with many restriction endonucleases in the chicken. This result suggests that the *Xho*I family repeating units compose higher repeating regions larger than 2.2Mb, perhaps about 3-5Mb. However, it is not sure whether these higher repeating regions are constituted only with the *Xho*I family, and whether the same constituent is detected with different restriction endonucleases. In order to clear these problems, it is necessary to perform hybridization with other W chromosome-specific repeating units and also double digestion with several endonucleases.

Several *Dra*I and *Eco*RI fragments from the W chromosomal DNAs, which did not hybridize with the 0.7Kb *Xho*I repeating units, were found by comparison of the hybridization signals and the bands in ethidium bromide staining (Fig. 3). This result

indicates that these *Dra*I and *Eco*RI fragments never contain the *Xho*I family sequences. SAITOH *et al.* (1991) found the W chromosome-specific 1.2 Kb *Eco*RI repeating units and performed pulsed field analysis of large restriction fragments containing *Xho*I or *Eco*RI family sequences. The *Eco*RI family sequences do not hybridized PFGE fragments in digestion with *Eco*RI in the range greater than a few hundred kilo-basepairs. The 1.2 Kb *Eco*RI repeating units further contained a restriction site with *Dra*I. Consequently, it is considered that the detected *Dra*I and *Eco*RI fragments in the present study do not contain both *Xho*I and *Eco*RI family sequences, and the fragments may be occupied by other repetitive sequences or unknown DNA sequences.

The karyotype of the chicken and quail possesses 78 chromosomes and many of chromosomes are microchromosomes which are much smaller than macrochromosomes. As shown in Fig. 4, the PFGE pattern of the intact genomic DNAs of the chicken revealed 2 bands of about 2Mb. However the 2 bands were not hybridized with the *Xho*I family sequences and most of signals were appeared in origin. The pattern of the quail was similar to that of the chicken. Therefore it is supposed that the 2 bands separated from the intact genomic DNAs of the chicken and quail by PFGE correspond to microchromosomes.

Fig. 4. Separation of the intact genomic DNAs of Japanese Game chickens by PFGE on 1% agarose gel. Arrows show bands which are supposed to be the microchromosomal DNAs. Lane M contains DNA size marker, chromosome-sized DNAs of *Saccharomyces cerevisiae*.

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家禽における W 染色体高分子 DNA のパルス フィールドゲル電気泳動による解析

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パルスフィールドゲル電気泳動を用いてニワトリおよびニホンウズラの W 染色体 DNA 分子をアガロースゲル中でバンドとして分離し、ニワトリの W 染色体特異的な反復 DNA 配列をプローブとして W 染色体の DNA 構成を研究した。

DNA 試料は赤血球からゲルブロック法により調製した。パルスフィールドゲル電気泳動後、エチジウムブロマイド染色によりニワトリおよびニホンウズラにおいて数百 Kb から数 Mb (数千 Kb) の範囲でバンドとして明瞭な分離パターンが検出された。そのうち、W 染色体 DNA に由来するバンドは 1 ペアーの親とそれらから得た F₁ および F₂ 個体の雌雄のパターンの比較により判定した。その結果、ニワトリでは 20 種類の制限酵素のうち 14 種類において W 染色体 DNA に由来するバンドが 1～8 本検出され、それらのバンドの多くは 2.2 Mb を超える巨大な DNA の断片であった。しかし、ニホンウズラでは明瞭な W 染色体 DNA に由来するバンドは検出されず、W 染色体の DNA 構成においてニワトリとかなり異なっていると考えられた。

W 染色体 DNA に由来するバンドを判定した後、ニワ

トリの W 染色体特異的な反復 DNA 配列をプローブとしてケミルミネセンスによるサザンハイブリダイゼーションを行った。その結果、ニワトリにおいて 16 種類の制限酵素で 3～5 Mb の範囲に 1～3 本のハイブリダイゼーションシグナルがほぼ共通して検出され、W 染色体特異的な反復 DNA 配列が 3～5 Mb もの巨大なサイズの高度反復領域を形成していることが示唆された。制限酵素 *Hind*III, *Pst*I および *Xba*I ではハイブリダイゼーションシグナルがそれぞれ 9 本、7 本および 9 本と多数検出され、品種、系統の比較に有効であると考えられた。また、制限酵素 *Dra*I および *Eco*RI ではハイブリダイゼーションシグナルが対応していない W 染色体 DNA に由来するバンドが 3 本および 5 本検出され、これらのバンドは現在報告されている W 染色体特異的な反復 DNA 配列を含んでいない DNA 領域であると考えられた。

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