Sequence characterization of K-gene linked region in various chicken breeds
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The avian endogenous virus gene (ev21) and dominant sex-linked late feathering (LF) gene (K) are closely associated on the Z chromosome of LF chickens. The gene is linked to two large repeats: ev21-unoccupied repeat region (URa) and ev21-occupied repeat region (OR). On the other hand, the recessive allele (early feathering, EF: k−) is linked to ev21-unoccupied region (URb). These three regions show high similarity but due to minor sequence differences, restriction enzyme treatment distinguishes URa and URb in White Leghorn. However, the applicability of the PCR-RFLP technique or the association between ev21 and K gene are not well investigated in the other breeds or strains. In this study, loci were detected in White Leghorn, Nagoya, Silky, Geline but not Gifujidori breeds of chickens, and the URa and URb from the various breeds were sequenced. Four types of sequence (URa- and URa- from LF or positive, URb- and URb- from EF or negative) were identified. At position 1072, URa- and URa- had “GGCC” whereas URa- had “AGCC”. The transition at clearly indicates that the PCR-RFLP technique used for White Leghorn is not applicable to Nagoya. Interestingly, both URa- and URa- were obtained from the positive sample of Geline. Presence of different sequence of URa in the Geline may explain that fixation of one of sequences to White Leghorn or Nagoya had occurred during breed establishment.

Key words: chicken, feathering, k gene, sexing


Introduction

Due to its prolific reproductive rate, White Leghorn is the dominant chicken used for production of table eggs in many parts of the world. Selection of females has been greatly facilitated by introduction of the sex-linked late feathering (LF) gene “Kurz” (K) into the maternal strain. Since the LF trait is dominant to early feathering (EF) (Warren, 1925), mating LF female to EF male only produce EF female progeny. Thus, the feathering trait makes it possible to rapidly identify the sexes and reduce costs associated with the manual sexing of 1-day chicks.

The avian endogenous virus gene (ev21) and K gene are closely associated on the Z chromosome of LF chicken (Bacon et al., 1988). Based on progeny testing, the linkage distance between K gene and ev21 was estimated at less than 0.3 cM (Smith and Fadly, 1994). Hence, the strong linkage between K gene and ev21 makes it possible to identify chickens carrying the K gene by PCR (Iraqi and Smith, 1994).

The genetic component of ev21-K complex locus has been further investigated using site-specific probes. Levin and Smith (1990) show the presence of additional DNA regions homologous to DNA sequences flanking the ev21 integration site. Furthermore, Iraqi and Smith (1995) confirmed that ev21 integrated into one of the two large homologous elements on the Z chromosome of LF White Leghorn chickens. Since, K gene and ev21 were closely related, Smith and Levin (1991) classified the three sequences; ev21 integrated site (occupied repeat: OR), homologous DNA region produced by duplication (unoccupied repeat: URa), and non-ev21 integrated DNA regions (un-occupied repeat: URb).

Fixing the genotype of maternal strain and paternal strain into homozygous populations is the first step to apply sexing using feathering. Although phenotypic differences between birds homozygous and heterozygous for K gene exist (Siegel et al., 1957), it is very hard to distinguish small differences due to the expression of LF by both homozygous and heterozygous K gene chickens. However, sequence differences between URa and URb...
have been exploited to develop RFLP genotyping method in White Leghorn (Iraqi and Smith, 1994).

Sexing using LF and EF was commercially applied in the White Leghorn. The presence of LF or ev21 was reported in other strains or breeds (Levin and Smith, 1990; Smith and Levin, 1991). However, it is unknown whether the RFLP method developed for White Leghorns to distinguish between K/K and K/k loci is applicable to other strains or breeds. Moreover, the association between the K gene and ev21 in other breeds or strains has not been delineated. Since developing the RFLP method applicable to other strains or breeds gives big advantage to poultry industry, identification and/or characterization of URa and URb in the other strains or breeds is very important to establish the phenotypically fixed strain. Accordingly, this study was primarily aimed to investigate the possibility of carrying ev21 in the population of several breeds and secondarily characterize the sequence of OR, URa and URb in the various breeds.

Materials and Methods

White Leghorn (n = 10 for EF and n = 10 for LF), Nagoya (n = 10 for EF and n = 10 for LF), Silky (n = 73) (maintained at Aichi Agricultural Research Center), Gifujidori (n = 87) (maintained at Gifu Prefectural Livestock Research Institute) and Geline (n = 63) (maintained at INRA Nouzilly) were used in this study. Samples of White Leghorn and Nagoya were obtained from closed flocks, which were phenotypically fixed and established strains for feathering to investigate the association between ev21 and feathering. Silky, Gifujidori and Geline were used to examine the presence of ev21 positive individuals in the population of breed. To identify the sequence and genotype, female sample of Silky, Gifujidori and Geline was used. Genomic DNA was extracted from red blood cells as previously described (Kansaku et al., 2005).

Characterization of ev21 Carrying Individuals

Primers GS-10 (CCTAGAACACTGGACATGGTAGATA TCTCAGGCC) and GS-13 (GGTGTCACCTGGGTG TAGATGAGCA) originally developed by Iraqi and Smith (1994) were used for identification of ev21 carrying individuals. Genomic DNA (0.1μg) was subjected to 35 cycles of PCR amplification using Ex-Taq polymerase (Takara, Shiga, Japan) in a total volume 25μl. The amplification profile consisted of 30 sec of denaturation at 98°C for the first cycle and 1 sec per cycle thereafter, 15 sec annealing at 59°C, and 15 sec extension at 72°C for the first 24 cycles and 2 min extension on the final cycle.

PCR Amplification of K Gene Linked Region

Genomic DNA samples identified as ev21 carrying were used for PCR amplification of K gene linked region. Primers (GS-9: AATGGTACTACAGAGAAGGTAGGTA TCTCAG, GS-23: GTAAAGACTAACACAGTATTCTC GAGT) developed by Iraqi and Smith (1994) was used for PCR. The location of the primers and the sequencing strategy are indicated in Figure 1. The amplification profile consisted of 1 min of denaturation at 98°C for the first cycle and 1 sec per cycle thereafter, 1 min annealing at 55°C, and 1 min extension at 72°C for the first 40 cycles and 2 min extension on the final cycle. PCR products were purified (Rapid PCR Purification system; Origene Technologies, Rockville, MD, USA) and used for direct sequencing. Based on the sequence identified in this study, primers (NGK1: CTGAGATATCATACATGTGC, NGK-2: CTCACACTACTGCAAGAAG, NGK 3: CCAAACACTTGTATATGG, NGK4: GCAGAGGTTCGAGAAGAC, NGK5: TCAGATCC AAGTGTCTGAGG) were designed for characterization of sequence of PCR products. DNA sequencing was performed using an Applied Biosystem Model 310 sequencer and the dideoxy-mediated chain-termination method (Sanger et al., 1977).

Confirmation of Presence of Duplicated Sequence

To confirm the presence of OR and URa in the ev21 positive individuals different combinations of primers (NGK1 and GS23, and GS13 and GS23) were used to amplify OR and URa sequence. PCR products were purified and directly sequenced as previously described.

Results

All samples of Nagoya and White Leghorn were sequenced. In the sample of Silky and Geline, 13 out of 73 and 58 out of 63 samples were ev21 positive, respectively. Among the ev21 positive sample, all samples of Silky and a randomly selected sample (n = 24) of Geline were sequenced. However, no ev21 positive sample was detected from Gifujidori hen (n = 87). PCR products from ev21 negative birds of all breeds were sequenced to compare and identify the differences between ev21 positive and negative individuals. Sequencing results of PCR products amplified between GS9 and GS23 were summarized (Table 1). Genetic variations were detected at positions 126, 189~193, 213, 294, 301, 307, 333, 474, 496, 506, 514, 557, 616, 694, 738, 754, 756~757, 794, 1063~1070, and 1072 (Fig. 2). At positions 126, 213, 294, 307, 474, 616, 789 and 1072, transition was detected, whereas at positions 333, 506, 514, 557, 694 and 794 transversion was detected. At positions 189~193, 301, 754, 756~757, and 1063~1070, insertion or deletion was detected. Thus, total 19 points of difference were detected. A total of 4 types of sequence (URa-1 and URa-2 from LF or ev21 positive, URb-1 and URb-2 from LF or ev21 negative) were identified. The sequence URa-1 was detected from White Leghorn, Silky and part of Geline, whereas URa-2 from rest of Geline and Nagoya. Thus, ev21 positive Geline showed URa-1 or URa-2. The sequence URb-1 was observed from Gifujidori, Nagoya, Silky, and White Leghorn. However, URb-2 was observed only from Geline. To analyze the similarity of sequences, a phylogenetic tree was constructed (Fig. 3).

Since LF carrying ev21 integrated region (OR) and duplicated non-integrated region (URa) derived from common origin, the primer pair NGK1 and GS-23 was
Fig. 1. Primer location and sequencing strategy for $K$ gene linked region of the chicken $Z$ chromosome.

Table 1. Summary of the $K$ gene linked region

<table>
<thead>
<tr>
<th>Breed</th>
<th>Phenotype</th>
<th>$ev_{21}$</th>
<th>Type of sequence</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geline</td>
<td>Unknown</td>
<td>+</td>
<td>URa-2</td>
<td>0.767</td>
</tr>
<tr>
<td>Geline</td>
<td>Unknown</td>
<td>+</td>
<td>URa-1</td>
<td>0.154</td>
</tr>
<tr>
<td>Geline</td>
<td>Unknown</td>
<td></td>
<td>URb-2</td>
<td>0.079</td>
</tr>
<tr>
<td>Silky</td>
<td>Unknown</td>
<td>+</td>
<td>URa-1</td>
<td>0.180</td>
</tr>
<tr>
<td>Silky</td>
<td>Unknown</td>
<td></td>
<td>URb-1</td>
<td>0.820</td>
</tr>
<tr>
<td>White Leghorn</td>
<td>LF</td>
<td>+</td>
<td>URa-1</td>
<td>Not done</td>
</tr>
<tr>
<td>White Leghorn</td>
<td>EF</td>
<td></td>
<td>URb-1</td>
<td>Not done</td>
</tr>
<tr>
<td>Nagoya</td>
<td>LF</td>
<td>+</td>
<td>URa-2</td>
<td>Not done</td>
</tr>
<tr>
<td>Nagoya</td>
<td>EF</td>
<td></td>
<td>URb-1</td>
<td>Not done</td>
</tr>
<tr>
<td>Gifujidori</td>
<td>Unknown</td>
<td></td>
<td>URb-1</td>
<td>1.000</td>
</tr>
</tbody>
</table>

used to amplify two products originating in OR and URa. To investigate the sequence similarity between OR and URa, PCR products between GS-9 and GS-23, and NGK 1 and GS-23 were also sequenced. Simultaneous base signal “T/C”, “A/C” and “T/C” were detected at positions 474, 514, and 557 respectively, from LF Nagoya and most of $ev_{21}$ positive Geline (Fig. 4). However, no simultaneous signals were detected from LF White Leghorn, Silky and part of $ev_{21}$ positive Geline. Bases of homologous positions were “C”, “C” and “G” at positions 474, 514, and 557 respectively. Likewise, “C”, “C” and “G” at positions 474, 514, and 557 were detected from $ev_{21}$ negative samples.

Discussion

This study clearly demonstrated sequence diversities of the URa and URb in the various chicken breeds. Especially, the sequence of URa and URb in the restriction en-
Fig. 2. Sequence alignment of URa-1, URa-2, URb-1 and URb-2. Regions (1~120 and 1081~1456) were removed due to the completely identity. URa-1: sequence from LF White Leghorn, ev21 positive Silky and part of Geline. URa-2: Sequence from LF Nagoya, ev21 positive Geline. URb-1: Sequence from EF White Leghorn and Nagoya, ev21 negative Silky and Gifujidori. URb-2: Sequence from ev21 negative Geline. Dots indicate nucleotides identical to URa-1. Uppercase letter and dash represents difference and insertion or deletion among the sequences, respectively.
zyme (Hae III) recognition site is worthy of notice. At position 1072–1075, URa-2, URb-1, and URb-2 had “GGCC” whereas URa-1 had “AGCC”. Interestingly, the population of ev21 positive Geline contains both URa-1 and URa-2 (URa-2: URa-1 = 5:1). This result may indicate that two types of sequence of URa were fixed: URa-2 in the population of Nagoya whereas URa-1 in that of White Leghorn. Moreover, PCR-RFLP technique developed to distinguish between the URa-1 and URb-1 of the White Leghorn was applicable to Silky and part of Geline, but not applicable to Nagoya and most of Geline. On the other hand, the base at position 294 of URb-1 of the White Leghorn, Nagoya, and Silky was ‘T’ whereas URb-2 of the Geline was ‘C’. Sequence of position 292 to 295 was “GACC” in the URa-1, URa-2 and URb-2, whereas “GATC” in the URb-1. Thus, PCR-RFLP technique using Mbo I for cutting the sequence “GATC” is applicable to distinguish the URa-2 and URb-1 in Nagoya.

Although the feathering phenotype of White Leghorn

![Phylogenetic tree of K gene linked region. Tree was prepared by NJ method. Genetic distance of each branch was shown.](image)

**Fig. 3. Phylogenetic tree of K gene linked region.** Tree was prepared by NJ method. Genetic distance of each branch was shown.

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(A)  
![Sequence flurogram of PCR products.](image)

Position 470–478  
GGTA (C/T)AACA

(B)  
![Sequence flurogram of PCR products.](image)

Position 510–518  
GTAA (C/A)ATCC

(C)  
![Sequence flurogram of PCR products.](image)

Position 553–561  
GTAC (T/G)GCAA

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**Fig. 4. Sequence flurogram of PCR products.** (A): PCR products by primers GS-9 and GS-23 from LF White Leghorn. (B): PCR products by primers GS-9 and GS-23 from LF Nagoya. (C): PCR products by NGK1 and GS23 from LF Nagoya and most of part of ev21 positive Geline. Primer pair NGK1 and GS-23 amplified two products originating in OR and URa. Based on the PCR products between GS-9 and GS-23, positions were expressed in this figure.
and Nagoya was determined before the study, the phenotype of Geline and Silky was not known. Thus, it is still unknown whether Geline and Silky carry the EF and LF trait. Both in White Leghorn and Silky, the sequence of URa and URb was fixed to URa-1 and URb-1, respectively. This may suggest the presence of EF and LF in the Silky. On the other hand, the sequence of the URb-2 of the Geline showed distant relationship to URb-1 of the White Leghorn, Nagoya and Silky. Interestingly, phylogenetic analysis constructed using NJ method revealed close relationship to URa-1 of the White Leghorn (Fig. 1). This may indicate that mutation and/or recombination of the Z chromosome or reversion occurred after breed establishment. Since Levin and Smith (1990) reported the EF phenotype with ev21 positive chickens, ev21 is not the sole determinant of the LF. Furthermore, the presence of ev21 without URa could be found in EF in various chicken strains (Boulliou et al., 1992). Thus, further analysis of association between ev21 and phenotype of feathering is required.

Sequencing of PCR products between GS-13 and GS-23 from ev21 positive samples showed C, C, and G at positions 474, 514 and 557 of GS-9 and GS-23. Since both OR and URa contain the region between NGK1 and GS-23, PCR produces and amplifies product which originates from different regions. LF White Leghorn, Silky and part of ev21 positive Geline showed complete identity between PCR product of OR origin and URa-1 origin. However, LF Nagoya and most of ev21 positive Geline showed slight differences between OR and URa-2. These bases explain the simultaneous base signal detected in PCR products between NGK1 and GS-23 from LF Nagoya and most of ev21 positive Geline (Fig. 4C). The reason for differences at these positions is unknown. The sequence amplified between GS-9 and GS-23 from ev21 negative sample may provide new insight. All ev21 negative samples except Geline showed T, A, T at positions 474, 514 and 557. These results may indicate that ev21 negative sample reflect the original sequence, whereas sequence identified in the White Leghorn or ev21 negative Geline appeared later by mutation or chromosome recombination.

In conclusion, the PCR-RFLP technique developed for the genotyping of the White Leghorn is applicable to some breeds and sequencing of the URa and URb may provide useful information for identification of the genotype.

Acknowledgments

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References


