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Microsatellite Polymorphism in Commercial Broiler and Layer Lines Estimated Using Pooled Blood Samples

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ABSTRACT For 17 microsatellite markers, allele frequencies were determined in nine highly selected commercial broiler and six highly selected commercial layer lines using pooled blood samples from 60 animals. The average number of marker alleles was 5.8 over all lines, 5.2 over broiler lines, and 3.0 over layer lines. The average number of marker alleles within a line was 2.9, 3.6, and 2.0 for all, broiler, and layer lines, respectively. Over all 15 lines, the average percentage of heterozygosity was 42, whereas the heterozygosity in the broiler lines was 53% and in the layer lines only 27%. In broiler lines, 50% of the marker-line combinations showed a heterozygosity above 60%, whereas this was only 5% in layer lines. Estimation of allele frequencies with microsatellite markers was first assessed in pooled and individual samples before usage in the commercial lines. Allele frequencies for 19 microsatellite markers were estimated in chicken pooled blood samples and compared with allele frequencies from individual typed animals. Similar results were obtained when pooled blood samples (heterozygosity of 35.3%) or individual typed animals (heterozygosity of 34.2%) were used. The method to determine allele frequencies using pooled blood samples is faster, cheaper, and as reliable and repeatable as determining allele frequencies using individual typings.

(Key words: chicken, microsatellite markers, pooled blood samples, heterozygosity, population screening)

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INTRODUCTION

A marker is an identified genomic site and marker alleles represent polymorphisms at this site. Markers can be used in animal breeding programs: e.g., in marker-assisted selection (Soller, 1978) or marker-assisted introgression (Hillel et al., 1990). Marker-assisted selection exploits marker-quantitative trait loci (QTL) associations. Due to linkage disequilibrium, the marker illustrates part of the within-family variance, thus providing additional information for selection purposes (Lande and Thompson, 1989). Marker-QTL associations can be determined by association studies in resource populations. For this purpose, the establishment of a marker map from linkage studies in reference populations is useful. The degree of marker polymorphism, i.e., the number of alleles and the percentage of heterozygous animals, determines the number of informative gametes per animal used to estimate associations and linkages (Van der Beek and Van Arendonk, 1993). High marker polymorphism within a line or population under selection will enhance marker-assisted selection (Dekkers and Dentine, 1990).

Marker polymorphism is used to determine genetic variation within and between breeds, i.e. genetic distance, heterozygosity and inbreeding (Nei, 1987; Kuhnlein et al., 1989, 1990; Lynch, 1990; Groen, 1993). Genetic distance might be used to predict heterosis (Gloeck, 1974; Goddard and Ahmed, 1982).

Different types of locus specific markers are available, i.e., minisatellite, microsatellite, random amplified polymorphic DNA (RAPD), and restricted fragment length polymorphism (RFLP). The application of microsatellite markers is currently thought to be useful as they are numerous and randomly distributed in the genome, they seem highly polymorphic, and they show codominant inheritance. More than 200 polymorphic chicken microsatellite markers have been mapped on the chicken genome (Khatib et al., 1993; Cheng and Crittenden, 1994; Crooijmans et al., 1994, 1995, 1996).

Allele frequencies have been determined for microsatellite markers using individual typings in human (Bowcock et al., 1994) and pigs (Van Zeveren et al., 1995). Allele frequencies with microsatellite markers can also be estimated from a pooled sample (Facek et al., 1993; Khatib et al., 1994; LeDuc et al., 1995). In these studies, individually isolated DNA samples were pooled. Alternatively, blood could be first pooled, after which DNA is isolated from the pooled blood sample.
The aim of this research was to test the use of microsatellite markers on pooled blood samples, and to use this technique to describe microsatellite polymorphism in highly selected commercial layer and broiler lines.

**MATERIALS AND METHODS**

**Microsatellite Markers and PCR Conditions**

The microsatellite markers used have been described by Crooijmans et al. (1993, 1994, 1995, 1996). All markers used contain a (TG) repeat except for MCW41, MCW42, and MCW43, which contain a poly (T) repeat. The PCR reactions were carried out in a total volume of 20 μL containing 10 to 100 ng genomic DNA, 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH = 8.3, 1 mM tetramethylammoniumchloride (TMAC), 0.1% Triton X-100, 0.01% gelation, 200 μM dNTP, 0.5 Unit Tth DNA polymerase¹ and 295 nM of each primer, one of which was labeled with the fluorescent dye Fluorprime² at the 5’ end. The PCR reaction consisted of 5 min at 95 C followed by 35 cycles of PCR, each cycle consisting of 30 s at 94 C, 45 s at 55 C, and 90 s at 72 C, and finally followed by an extension step of 10 min at 72 C. A mixture of 1 μL PCR amplification product and 80% formamide was made, denatured by heating to 94 C for 5 min, and resolved on a 6% denaturing polyacrylamide gel, Sequagel-6.³ Analyses were performed using an Automated Laser Fluorescent (ALF) DNA Sequencer.² The size of the detected alleles was calculated using the computer program Fragment Manager.² Allele frequencies were estimated from relative (to total peak area within the sample of the line) fluorescent areas.

**Analyses**

Two sets of animals were used, one for testing the method of estimating the allele frequency in individual versus pooled samples, and a second for estimating allele frequencies in commercial broiler and layer lines.

First, 19 markers were tested on DNA isolated from individual blood samples from 27 males of two different commercial White Leghorn lines, and on DNA isolated from two different pooled blood samples of the 27 individuals (equal amount of blood from each individual was pooled). The 27 males are descendants from two different layer lines (12 and 15 animals). Concentrations of DNA in pooled and individual samples were adjusted to 10 ng/μL. The PCR on the two pooled samples and calculation of allele frequencies were performed in duplicate, and repeated (in duplicate) another day. The number of alleles observed per marker was determined. Heterozygosity was calculated per marker, according to Nei (1987):

\[
H_i = \frac{0}{2n/(2n-1)}[1 - \sum m_i(p_i)^2],
\]

assuming Hardy-Weinberg equilibrium, where \(n\) is the number of animals in the sample (27 in this study), \(m_i\) is the number of alleles at locus \(i\), and \(p_i\) is the frequency of the \(i\)th allele at locus \(i\). This allele frequency can either be computed from individual typings or from the analyses of the pooled sample.

Secondly, blood samples of 60 individuals per line (20 and 40 randomly chosen males and females) were pooled, and genomic DNA was isolated from these pooled samples. Nine broiler (Cornish and Rock) and six layer (White Leghorn) lines were sampled. Analyses were performed in duplicate, starting with two different pooled blood samples. Seventeen markers were analyzed in all lines. The number of alleles observed per marker was determined for all lines, for broiler lines, and for layer lines. Moreover, the number of alleles per maker per line, averaged for all lines, for broiler lines, and for layer lines was determined. Heterozygosity was calculated per line per marker, according to Nei (1987), assuming Hardy-Weinberg equilibrium, where the number of animals in the sample was 60. Heterozygosity per marker was averaged for all lines, for broiler lines, and for layer lines.

**RESULTS**

**Comparison of Individual Sampling with Pooled Sampling**

Allele frequencies of 19 microsatellite markers used for the 27 individual animals and for the pooled sample are shown in Figure 1. Markers MCW3 and MCW19 were monomorphic. The number of alleles observed in the 27 individual animals from the two layer lines for these 19 markers varied from 1 to 6 (Table 1). The heterozygosity for the 19 markers calculated from the individual typings varied from 0 to 79.7%, with an average of 35.3%. The average heterozygosity in the pooled samples was 34.2%. The average absolute difference in heterozygosity between individual and pooled samples was 34.2%. A large difference was observed for marker MCW1 only, for which the heterozygosity was calculated to be 40.1% for the individual samples and 21.1% for the pooled blood samples. For all the other markers, the difference in heterozygosity between individual and pooled samples varied from 0 to 8.8% with an average of 2.4%.

**Marker Polymorphism for 15 Commercial Chicken Lines**

The pooled samples were typed for a total of 17 markers. For markers MCW22 and MCW43, it was not possible to determine the allele frequency in the broiler

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lines because of too much interference of the many marker alleles with the stutter peaks. The number of alleles per marker for all lines averaged 5.8 (Table 2) with a maximum of 12 and a minimum of 2. Within a line, the average number of alleles per marker was 2.9. One microsatellite marker (MCW19) was monomorphic in the layer lines but polymorphic in the broiler lines. Markers MCW16 and MCW18 each had 8 alleles in one broiler line. The percentage of heterozygous animals for a single marker was 42 averaged over all lines, and 53 and 27 averaged for broiler and layer lines, respectively. In total, 153 and 102 marker-line combinations were tested in broilers and layers, respectively. Figure 2 shows the proportion of marker-line combinations above a given level of heterozygosity. For example, in broilers 50% of the marker-line combinations had a heterozygosity above 60%, and 25% had a heterozygosity above 70%. In layers, only 5% of the marker-line combinations had a heterozygosity higher than 60%.

**DISCUSSION**

Estimating allele frequencies from one pooled sample is faster and cheaper than estimating allele frequencies from many individual samples. We show that the method using a pooled sample is useful compared with the method using individual typings. Estimation of the allele frequencies from pooled samples has been described previously (Pacek et al., 1993; LeDuc et al., 1995; Khatib et al., 1994). However, in those studies, DNA was pooled instead of blood, whereas we pooled the blood from the individual animals and subsequently isolated the DNA directly from these pooled blood samples. Because in birds both red and white blood cells contain nuclei, the number of nuclei containing cells per milliliter of blood is rather constant for a given sex and stage of development. Therefore, pooling of equal amounts of blood per sample is a feasible alternative to individual sampling, which is contrary to mammals, in which large differences can occur in the number of nuclei-containing white blood cells. In these cases adjustments in the amount of blood have to be made. The method to determine the allele frequency with microsatellite markers in poultry such as performed by Khatib et al. (1994), by densitometry of autoradiograms is difficult to compare with the fluorescent method that we used. The use of automated sequencers has made it easier to analyze large numbers of samples and quantitative information for the different marker alleles is directly available. The combination of pooled blood samples and fluorescent analysis makes this method very powerful.

An artifact typically generated by PCR amplification of microsatellites is stutter peaks. These are PCR products smaller than the major amplification product, generally by increments of the repeat unit, and each amplified allele typically shows these stutter bands (Le Duc et al., 1995). In chicken, stuttering of alleles is rare compared with that found in many other species,
CHICKEN MICROSATELLITE POLYMORPHISM

TABLE 1. Microsatellite polymorphism in 27 animals either based on individual samples or on pooled samples

<table>
<thead>
<tr>
<th>Marker</th>
<th>Total number of alleles observed</th>
<th>Percentage heterozygosity1</th>
<th>Absolute difference3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCW1</td>
<td>3</td>
<td>40.1 (± 2.9)</td>
<td>19.0</td>
</tr>
<tr>
<td>MCW2</td>
<td>2</td>
<td>50.9 (± 0.3)</td>
<td>0.8</td>
</tr>
<tr>
<td>MCW3</td>
<td>1</td>
<td>0.0 (± 0.0)</td>
<td>0.0</td>
</tr>
<tr>
<td>MCW4</td>
<td>3</td>
<td>62.3 (± 2.5)</td>
<td>5.6</td>
</tr>
<tr>
<td>MCW5</td>
<td>6</td>
<td>79.7 (± 0.4)</td>
<td>1.3</td>
</tr>
<tr>
<td>MCW7</td>
<td>3</td>
<td>31.3 (± 4.7)</td>
<td>4.8</td>
</tr>
<tr>
<td>MCW9</td>
<td>2</td>
<td>50.9 (± 0.1)</td>
<td>0.0</td>
</tr>
<tr>
<td>MCW14</td>
<td>2</td>
<td>42.5 (± 1.3)</td>
<td>1.5</td>
</tr>
<tr>
<td>MCW16</td>
<td>2</td>
<td>7.3 (± 2.0)</td>
<td>1.8</td>
</tr>
<tr>
<td>MCW18</td>
<td>2</td>
<td>3.8 (± 1.7)</td>
<td>0.3</td>
</tr>
<tr>
<td>MCW19</td>
<td>1</td>
<td>0.0 (± 0.0)</td>
<td>0.0</td>
</tr>
<tr>
<td>MCW22</td>
<td>3</td>
<td>50.1 (± 2.8)</td>
<td>6.5</td>
</tr>
<tr>
<td>MCW23</td>
<td>2</td>
<td>47.5 (± 2.6)</td>
<td>2.1</td>
</tr>
<tr>
<td>MCW34</td>
<td>3</td>
<td>52.0 (± 1.7)</td>
<td>3.3</td>
</tr>
<tr>
<td>MCW36</td>
<td>2</td>
<td>45.3 (± 0.5)</td>
<td>0.1</td>
</tr>
<tr>
<td>MCW39</td>
<td>3</td>
<td>26.7 (± 4.4)</td>
<td>4.9</td>
</tr>
<tr>
<td>MCW41</td>
<td>2</td>
<td>7.3 (± 1.3)</td>
<td>0.1</td>
</tr>
<tr>
<td>MCW42</td>
<td>3</td>
<td>34.3 (± 2.2)</td>
<td>1.2</td>
</tr>
<tr>
<td>MCW43</td>
<td>2</td>
<td>39.1 (± 1.1)</td>
<td>9.0</td>
</tr>
<tr>
<td>Average</td>
<td>2.5</td>
<td>35.3 (± 1.3)</td>
<td>3.4</td>
</tr>
</tbody>
</table>

1Heterozygosity calculated as $H = \frac{2n}{(2n-1)}[1-\sum(p_i^2)]$, assuming Hardy-Weinberg equilibrium, where $n$ is the number of alleles of 11.7 (Van Severen et al., 1994). Averaged over eight samples (two different pools typed in duplo and repeated on another day), with the standard deviation given between brackets (standard deviation calculated of the eight individual observations).

2 Absolute difference in heterozygosity between individual typings and pooled typings.

Although stutter bands has prevented calculation of the allele frequencies for MCW22 especially in the broiler lines. When stutter bands do occur, however, arithmetic corrections can be made on the allele frequency (LeDuc et al., 1995). Stuttering of alleles in mononucleotide repeat markers is a problem, especially when the marker alleles differ in length by only one base. This small difference happened with marker MCW43 in the broiler lines, where resolution of the gel matrix was not sufficient to allow distinction of the different alleles from the stutter bands. These resolution problems might be improved by using another type of gel matrix. One disadvantage of the use of a pooled sample compared with the use of individual samples is that the frequencies of genotypes can only be estimated from a pooled sample assuming the marker is in Hardy-Weinberg equilibrium. In our comparison, we used only a few animals (27 males from two different lines). The large sample size in farm animals that decrease the level of heterozygosity. Differences in the number of alleles larger: for pigs the average is 11.7 (7 loci), much higher than the 7.4 found in humans (30 loci) and 5.8 found in chickens (17 loci). In pigs and humans, more alleles resulted in a higher heterozygosity (Bowcock et al., 1994). The average number of alleles varied from 6.1 to 8.9. The results of heterozygosity in humans compared with pigs and chickens may be explained by strong selection and small population size in farm animals that decrease the level of heterozygosity. Differences in the number of alleles are large: for pigs the average is 11.7 (7 loci), much higher than the 7.4 found in humans (30 loci) and 5.8 found in chickens (17 loci). In pigs and humans, more alleles resulted in a higher heterozygosity (Bowcock et al., 1994; Van Severen et al., 1995), which agrees with our results. For example, marker MCW5 with 12 alleles observed in 15 lines yielded the highest heterozygosity (62%).

A moderate degree of polymorphism will limit the efficiency of marker-assisted selection when using microsatellite markers in farm animals. Selection of markers for use in a given line can increase the observed polymorphism. Markers can be highly polymorphic in one line and monomorphic in another line, which again emphasizes the need to select markers primarily based on polymorphism within the line used for a linkage or...
In conclusion, the observed polymorphism of microsatellite markers in commercial chicken lines tested is moderate. Fifty percent of the marker broiler combinations and 5% of the marker layer combinations showed a heterozygosity of more than 60%. These results should be considered when designing association and linkage studies and when estimating potential benefits from marker-assisted selection.

ACKNOWLEDGMENTS

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