Sex Identification of Newly Hatched Chicks by Fluorescence in situ Hybridization using a W-specific DNA Probe in Feather Follicle Cells

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The identification of the sex of chickens or chicken cells has biological and industrial significance. In the laboratory, molecular techniques have utilized W chromosome-specific repetitive DNA sequence fragments, such as Xho I or EcoRI family, for gender identification in chickens. The present study examined a rapid and reliable fluorescence in situ hybridization (FISH) procedure for chick sex identification using an easily obtainable sample: uncultured feather pulp cells. A chicken W chromosome-specific 416-bp partial DNA fragment of Xho I family was digoxigenin-labeled by polymerase chain reaction and used as an FISH probe for chick sex identification. In 60 newly hatched chicks, 28 of the chicks that were subsequently identified as females by the visual identification of ovaries exhibited a positive W-specific probe signal in their feather follicle cells; the others were negative for the W-specific probe signal and were identified as males by the presence of testes. Therefore, the FISH results were in perfect agreement with the anatomical analysis. We suggest that this FISH technique using feather follicle cells and the W-specific DNA probe will be useful for a fast, convenient and accurate sex diagnosis of newly hatched chicks.

Keywords: chick, feather, FISH, sex identification, W chromosome, Xho I family


Introduction

There are several benefits of the sex identification of day-old chicks, both for hatcheries and for the industry in general; the sexing of day-old chicks has reduced the cost of rearing by reducing labour and feed expenses. Currently, feather sexing and vent sexing are the representative sexing methods of day-old chicks. Feather sexing is based on the differences in the feather characteristics at hatching, because slow-feathering and fast-feathering are sex-linked traits. The gene for slow-feathering is dominant and controls the rate of wing and tail feathering in the chicks. The dominant slow-feathering characteristic (K-) is passed from mothers to their sons, and the rapid-feathering characteristic (kk) is inherited by daughters from their fathers. Thus, feather sexing can be used if the breed has both dominant and recessive feathering genes. However, most breeds of chickens do not possess these feather sexing characteristics, and feathering in both sexes appears identical. Vent or cloacal sexing relies on the visual identification of sex based on the appearance of sexual organs. In 1933, vent sexing was first published in Japan by professors Masui and Hashimoto, which was soon translated into English under the title ‘Sexing baby chicks’ (Martin, 1934). However, the art of vent or cloacal sexing of day-old chicks is difficult to master without instructions from an experienced sexer because the sexual organs of birds are located within the body and are not easily distinguishable. The copulatory organ of chicks can be identified as male or female by shape, but there are over fifteen different shapes to consider.

Sex identification in chickens or chicken cells also has biological significance. In the laboratory, the sex of chickens is easily identified by the karyotyping of cultured cells. Chicken sex chromosomes consist of ZZ in males or ZW in females, and the chromosomes have different shapes and are easily distinguishable (Krishan and Shoffner, 1966; Pollock and Fechheimer, 1976; Sohn et al., 2000). However, the karyotype analysis, which is performed on metaphase cells after several days of in vitro culture, is rather time-consuming for a routine lab procedure. In recent decades, the sex of chicken cells has been identified mostly by poly-
merase chain reaction (PCR) by targeting a W chromosome-specific repetitive DNA sequence, such as those generated by cleavage of \(Xho\) I or \(EcoRI\) restricted enzymes (\(Xho\) I or \(EcoRI\) family), which altogether almost occupies the total W chromosome DNA (Kodama et al., 1987; Saitoh et al., 1991; Klein and Ellendorff, 2000). Using PCR protocols, unique sequences, such as the chromo-helicase-DNA binding protein gene (CHD1) and a 0.6 kb \(EcoRI\) fragment (E0.6), also have been utilized for sex identification in chickens (Ogawa et al., 1997; Griffiths et al., 1998; Itoh et al., 2001). Most birds have two CHD sex-linked genes, one W-linked (CHD-W) and one Z-linked (CHD-Z). Using highly conserved primers flanking the intron of CHD1 and PCR amplification, females showed two fragments (CHD-W and CHD-Z), while males showed only one fragment (CHD-Z) clearly different size from the female-specific CHD-W fragment. PCR-based techniques using the CHD primers have been widely and successfully used to sex the non-ratite birds (Ellegren, 1996; Griffiths et al., 1996; Fridolfsson and Ellegren, 1999; Bermúdez-Húmaran et al., 2002; Hu et al., 2003; Ito et al., 2003; Takagi et al., 2007a, 2007b). Fluorescence in situ hybridization (FISH) using a W chromosome-specific DNA probe is another molecular cytogenetic technique for gender identification in chickens (Klein and Ellendorff, 2000; Sohn et al., 2002). FISH is used to directly detect and locate the presence of specific DNA sequences on chromosomes. The FISH sexing technique uses a W-specific DNA fluorescent probe that binds only to the W chromosome, regardless of cell types (Abinawanto et al., 1998; Sohn et al., 2002; Zhao et al., 2010). The identification of sex would be facilitated if tissues or cells could be readily obtained from day-old chicks. As the feather pulp of day-old chicks is an easily available sample, the present study was, thus, undertaken to examine a rapid FISH procedure for the identification of sex in newly hatched chicks using uncultured feather follicle cells.

Materials and Methods

Animals and Maintenance

Newly hatched chicks of Korean Native Chicken, Rhode Island Red and Black Cornish, which were hatched at the experimental farm of Gyeongnam National University of Science and Technology, Jinju, Korea, were used for the present study. For sampling, wing feathers were obtained from 20 newly hatched chicks of each breed. At the end of the lab experiment, all 60 chicks were sacrificed, and an anatomical inspection was performed for sex diagnosis. These chicks were cared for and handled according to the procedures of the institutional animal care and use committee (IACUC) of the university.

Preparation of Cells from Feather Pulps

Feather pulp was obtained from the newly hatched chicks by squeezing the content of the feather stalk with a pair of forceps (Fig. 1) into 15-ml conical tubes containing 3 ml of D-PBS (Gibco, Invitrogen Corp. Grand Island, NY, USA). The follicle cells were separated from the pulp by vigorous pipetting, followed by centrifugation for 10 min at 200 \(\times\) g. Precipitated cells were treated with 1.0% sodium citrate (Sigma Chem, St Louis, MO, USA) hypotonic solution and fixed in a methanol and acetic acid mixture (3:1 by volume). After repeating the above fixation procedure three times, the feather follicle cells were dropped onto glass slides and air-dried overnight.

Fluorescence In Situ Hybridization using a W-specific DNA Probe

A 416-bp DNA fragment was simultaneously labeled with digoxigenin (Dig)-dUTP (Roche, Mannheim, Germany) and amplified by PCR using the 5’-CCCAATATAAAGCT-CTTCAG-CTTCACT-3’ (22 mers) and 5’-GAAATGATTATTTCTGGGCACG-3’ (23 mers) primer pair and female chicken genomic DNA as the template, following the manufacturer’s instructions. The FISH procedure was slightly modified from those of Kobayashi et al. (1998) and Sohn et al. (2002). Briefly, the slides containing the feather follicle cells were incubated in RNase A (Sigma) and dehydrated in an increasing ethanol gradient. Hybridization solution (2 \(\times\) SSC, 50% formamide and 10% dextran sulfate, Roche) containing Dig-labeled probes was dropped onto the slides, and the samples were denatured at 72\(^\circ\)C for 10 min and hybridized at 38\(^\circ\)C for at least 1 hour. The slides were washed with 2 \(\times\) SSC at 72\(^\circ\)C and then with PN buffer (7 mM sodium phosphate, pH 8.0, containing 0.1% Nonidet 40, Roche) at room temperature. The air-dried slides were then incubated with an anti-dig-fluorescein isothiocyanate conjugate (FITC) at 38.5\(^\circ\)C for 10 min, washed with PN buffer, counter stained with propidium iodide (PI) and examined using a fluorescence microscope (Model AX-70, Olympus, Tokyo, Japan) at green (FITC) and red (PI) dual excitation wavelengths. The images were captured using a digital camera (DP-70, Olympus) and analyzed. At least 50 cells were evaluated for the W chromosome-specific signal in each specimen.

Results

To identify the sex of the chicks, we examined whether the W chromosome-specific DNA probe would bind to the chromosome in interphase nuclei derived from uncultured feather follicle cells of newly hatched chicks using the FISH procedure. Fig. 2 shows the representative nuclei of the
chick feather follicle cells using the W-specific DNA probe in which the W-specific DNA is indicated by green-yellow spots (FITC signals) against a red background of genomic DNA. The female feather follicle cells exhibited FITC signals via FISH using the W-specific DNA probe (Fig. 2a), whereas the male cells did not (Fig. 2b). Of the 60 hatched chicks, 28 exhibited the positive W-specific probe signal in their feather follicle cells (Table 1); these 28 chicks were subsequently identified as females by visual identification of the ovaries (Fig. 3a). The other 32 chicks, which produced

Table 1. Number of chicks analyzed by fluorescence in situ hybridization (FISH) using a W-specific DNA probe in feather follicle cells and by anatomical inspection for sex identification.

<table>
<thead>
<tr>
<th>Breeds</th>
<th>FISH result</th>
<th>Anatomical diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>W-probe signal</td>
<td>No signal</td>
</tr>
<tr>
<td>Korean Native Chicken</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Rhode Island Red</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Black Cornish</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>32</td>
</tr>
</tbody>
</table>

Fig. 2. Fluorescence in situ hybridization of chick feather follicle cells using a W-specific DNA probe. In female cells, one W-specific probe signal is shown in each nucleus (a), but no signals are observed in male cells (b).

Fig. 3. Sex identification of newly hatched chicks by the visual inspection of gonads. The female has both a degenerating and a developing ovary (a). The male has two developing testes (b).
no W-specific probe signal, were identified as males by the presence of testes (Fig. 3b). Therefore, the FISH results using the W-specific probe were in perfect agreement with the anatomical analysis.

Discussion

We targeted a 416-bp segment of the 717-bp Xho I family fragment (Kodama et al., 1987), which is the repeating unit of the W chromosome-specific repetitive DNA sequences in chicken, for the synthesis of a chicken W-specific FISH probe.

The chicken W chromosome is composed of repetitive DNA sequences, and Xho I and Eco R I families occupy 65% of its DNA (Saitoh and Mizuno, 1992; Suka et al., 1993). Digestion of the chicken genomic DNA with Xho I yielded two prominent female-specific fragments of 0.6 kb and 1.1 kb (Tone et al., 1982) that were specific for Gallus domesticus and were not found in other avian species. Furthermore, it was found that the 0.6-kb and 1.1-kb fragments, which comprised 46% of the total W chromosome, were repeated approximately 14,000 and 6,000 times, respectively (Tone et al., 1984). The 0.6-kb Xho I fragment has been cloned and extensively characterized by Kodama et al. (1987). It consists of 34 tandem repeats of 21 bp and, hence, its actual length is 717 bp. In the present study, the expected size of the PCR product, 416-bp of the W chromosome-specific partial Xho I family DNA fragment, was obtained from the genomic DNA of female chickens, regardless of the breed, i.e., Korean Native Chicken, Rhode Island Red and Black Cornish. The 416-bp sequence of the PCR product is shown in Fig. 4; this sequence exhibited a 400/416 (96%) identity with that of an Xho I family fragment deposited in GenBank (accession number X06548). We confirmed that this DNA segment is species specific and sex specific only for the Gallus domesticus W chromosome. Many researchers have previously reported the female-specific PCR-amplification of the Xho I family fragment in chickens using PCR primers of the same sequences used in this study (Clinton, 1994; Klein and Ellendorf, 2000; Sohn et al., 2002). However, one of these studies reported that the size of the PCR product was only 315 bp, which may be a mis-calculated value (Clinton, 1994). Klein and Ellendorf (2000) detected a 415-bp PCR product using the same primers with 1 ng to 10 ng of template DNA from female chickens and obtained a similar PCR product using approximately 10 ng of template DNA from males. In spite of the discrepancies between the amplification results, we obtained a consistent PCR product only from female DNA. We suggest that the composition of the PCR mixture, the amount of template DNA and the thermocycling conditions may be critical factors for the amplification of the Xho I family.

A 416-bp Dig-labeled DNA segment of the Xho I family was used as the FISH probe to detect the presence of the W chromosome in metaphases and interphase nuclei, and a signal on the W chromosome was detected using the metaphase chromosome spread (data not shown). The FISH probe also bound to the W chromosome of interphase nuclei in uncultured female cells, but not in male cells. Therefore the FISH procedure described here can omit the time-consuming cell culturing for obtaining metaphase chromosome spreads and directly use uncultured cells, without compromising the specificity of the procedure. The PCR methods in which a known chicken W chromosome-specific DNA sequence is amplified have been developed to diagnose sex (Clinton, 1994; Petitte and Kegelmeyer, 1995; Klein and Ellendorff, 2000), and such methods have an advantage in their rapid nature, requiring only several hours, and reliability in most cases. The PCR-based identification of sex, however, is not completely free of ambiguity or the mis-identification of a ZW or ZZ/chimeric karyotype. In addition, the PCR-based procedure cannot distinguish between the sex chromosomes when only a limited amount of DNA is available for PCR (Lee et al., 2003; Sato et al., 2003). Klein and Ellendorff (2000) evaluated the sexing accuracy of a PCR method in targeting an approximately 400-bp partial Xho I family fragment and performed FISH using the W-specific probe for sex diagnosis. They concluded that FISH is better than PCR in terms of the accuracy. Our results also indicate that the FISH method using a W-

**Fig. 4.** The nucleotide sequence of the 416-bp PCR product of the chick W chromosome-specific partial Xho I family DNA fragment. The shading indicates the w-5 and w-3 PCR primer sequences.
specific DNA probe containing the Xho I sequence is more reliable and accurate than PCR methods for the diagnosis of chick gender. Moreover, the FISH procedure saves time and trouble because the DNA purification of target cell is unnecessary.

In conclusion, a reliable chicken W chromosome-specific 416-bp DNA probe, which is a partial fragment of the 717-bp Xho I family repeat sequence, was isolated to identify sex in chicks. Using this probe, we employed a simple and reliable FISH technique for the sex-determination of chicks using readily available uncultured feather follicle cells. Therefore, we expect that this FISH technique will be useful for a fast, convenient and accurate sex diagnosis of newly hatched chicks for various scientific and industrial purposes.

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