RAPID COMMUNICATION

A simple and efficient method for extraction of PCR-amplifiable DNA from chicken eggshells

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Running title: DNA extraction from chicken eggshells

ABSTRACT

Recently, we reported a method for discriminating a Japanese brand of chicken, the Hinai-jidori. As an application of this method for discriminating Hinai-jidori eggs, we here report an efficient method for extracting maternal DNA from eggshells. Eggshell powder was completely decalcified with EDTA solution, and then DNA was isolated by conventional phenol-chloroform extraction and ethanol precipitation. The efficiency of DNA recovery from eggshells was 50-fold higher than that of a previously reported method. The recovered DNA could be used for polymerase chain reactions and 10 makers for identifying the Hinai-jidori chicken were detected. The genotypes of the Hinai-jidori exactly matched those of the Hinai-dori breed. Using this method, Hinai-jidori and Hinai-dori eggs could be distinguished from the eggs of Rhode Island Reds. This is the first report of a technique that can be used to discriminate the eggs of Hinai-jidori from those of other chickens, and it can also be utilized to validate the labeling of Hinai-jidori eggs in the market.

Key words: chicken, egg, brand discrimination, Hinai-jidori chicken, microsatellite markers.

INTRODUCTION

Food traceability is defined as the ability to follow particular foods through all stages of the food chain, from production to sale (Committee of the Guidelines for Introduction of Food Traceability Systems of Japan 2003), and its use is increasingly becoming standard practice throughout the agrifood industry. In this respect, DNA identification technology has been playing an important role in refining existing meat traceability systems since it ensures that products can be traced to the animal of origin.

The Hinai-dori is a chicken breed native to the Akita Prefecture of Japan. The Hinai-jidori, a cross between a Hinai-dori sire and a Rhode Island Red dam, has been commercialized and is a popular brand in Japan. However, in 2007, cases of the false labeling of Hinai-jidori chickens, including processed meat and eggs, came to light. Recently, we developed a method for discriminating the Hinai-jidori chicken from other chickens (Rikimaru & Takahashi 2007). It is relatively easy to extract DNA from meat using standard phenol-chloroform methodology; however, the extraction of DNA from eggs is of relatively limited applicability owing to the low yield of collected DNA (Strausberger & Ashley 2001). In addition, the eggs of the Hinai-jidori chicken cannot be distinguished from other pale brown eggs solely by outward appearance. Thus, DNA identification of Hinai-jidori eggs is also desirable. If a method for extracting DNA from eggshells with high yield could be developed, we would be able apply the method of Rikimaru & Takahashi (2007) for identifying Hinai-jidori eggs.

Here, accordingly, we describe an efficient method for extracting nuclear DNA from chicken eggshells. Using the DNA extracted from eggshells, we obtained microsatellite genotypes that can be used for the identification of Hinai-jidori eggs.

MATERIALS AND METHODS

Samples

Unfertilized eggs of the Hinai-dori (n = 8) and Rhode Island Red (n = 8) breeds were collected from the Livestock Experiment Station, Akita Prefectural Agriculture, Forestry and Fisheries Research Center. The unfertilized eggs of Hinai-jidori (n = 8) were collected from the Goto Farm (Kita-akita, Japan).

DNA extraction

Material on the surface of the eggs was sponged away under running water. The inner and outer shell membranes were removed, and the resultant eggshells were rinsed with sterile water and dried at room temperature. The eggshells were then physically powdered using a pestle and mortar. Forty milligrams of the eggshell powder was placed into a 1.5-ml microfuge tube, to which was added 800 μL of 0.5 M EDTA-2Na (ethylenediaminetetraacetic acid disodium salt, pH 8.0). The tube was placed in a shaker (M·BR-022; TAITEC, Tokyo, Japan) and incubated overnight at 56 °C with vigorous shaking (1400 rpm). Following the addition of 0.6 mL of phenol-chloroform, the tube contents were mixed by rotating the tube slowly on the wheel of rotator (RT-50, TAITEC) for 6 h. The tube was then centrifuged for 10 min and the upper layer transferred into a fresh 1.5-mL tube. Diethyl ether (0.6 mL) was then added and the tube was vortexed for 15 s. The tube was subsequently centrifuged for 1 min and the upper layer containing ether was removed. DNA was precipitated by the addition of 0.6 mL of ethanol, rinsed twice with 70% ethanol, and dissolved in 30 μL of deionized sterile water. The DNA was quantified using a spectrophotometer (GeneQuant Pro; GE Healthcare, Amersham, UK).

Microsatellite genotyping

Nine *ABR* markers (*ABR1003*, *ABR0241*, *ABR031*, *ABR1004*, *ABR1013*, *ABR0633*, *ABR1005*, *ABR0089*, and *ABR100*, Takahashi *et al*. 2005; Rikimaru & Takahashi

2007) and one *ADL* marker (*ADL0250*, Cheng *et al*. 1995) on chromosome Z exhibiting one fixed fragment size in the Hinai-dori breed (Rikimaru & Takahashi 2007) were genotyped. DNA was amplified in a 15-μL reaction volume, which contained 2.5 pmol of each primer for each marker, 200 μM of each dNTP, 0.8 mM $MgSO_4$, 0.5 units of KOD Plus polymerase (KOD-201; Toyobo, Tokyo, Japan), 1× reaction buffer provided by the supplier, and 10 ng genomic DNA, in a 96-well plate in an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Polymerase chain reaction (PCR) was performed as follows: a hot start of 2 min at 94 °C; 40 cycles of 15 s at 94 °C, 30 s at 58 °C, and 30 s at 68 °C; and a final elongation of 9 min 30 s at 68 °C. The PCR products were run with the internal size standard (GENESCAN 400HD [ROX] Size Standard; Perkin-Elmer, Foster City, CA, USA) in an ABI PRISMTM 3100 DNA Sequencer (Perkin-Elmer). The size of the fragments was analyzed using GeneScan (Version 3.7) and GeneMapper (Version 2.0) programs (Perkin-Elmer). Alleles were designated according to PCR product size, and allele frequencies were calculated directly from the observed genotypes.

RESULTS AND DISCUSSION

The DNA yields from 40 mg of eggshell ranged from 498 to 1689 ng (Table 1), with an average of 813 ng per sample. The sample yield per milligram of eggshell ranged from 12.5 to 42.2 ng. The extraction of DNA from eggshells was not considered possible until the method of Strausberger & Ashley (2001) was reported. Strausberger & Ashley (2001) obtained DNA yields ranging from15 to 90 ng from 50 mg eggshell of the brown-headed cowbird, *Molothrus ater*. The yield per milligram eggshell ranged from 0.3 to 1.8 ng. The efficiency of DNA recovery from eggshells in the current study was 50-fold higher than that obtained by Strausberger & Ashley (2001). The key point of our protocol is that eggshells are completely decalcified with EDTA solution, whereas Strausberger & Ashley (2001) digested eggshell powder using proteinase K without decalcification. EDTA does not only decalcify the eggshell but also protects DNA from the action of nucleases that require metallic ions, e.g., Mg^{2+} and Mn^{2+}, for activation. Consequently, the high efficiency of DNA recovery considerably reduces the chance of contamination in the subsequent PCR, and also facilitates analysis of a number of markers.

In the present study, all the DNA samples obtained from the eggshells were successfully amplified by PCR for 10 markers on the Z chromosome. The 10 markers revealed one fixed allele each in the Hinai-dori and Hinai-jidori eggs (Table 1). The allele size of each marker was consistent with that reported by Rikimaru & Takahashi (2007). It was found to be easy to distinguish between the eggs Hinai-dori and Rhode Island Red chickens and between those of Hinai-jidori and Rhode Island Red chickens. Our results suggest that the DNA embedded in eggshells was derived from the egg-laying female since the genotypes of the Hinai-dori eggs correspond to those of the Hinai-dori breed, as described previously (Rikimaru & Takahashi 2007). As expected, the genotypes of the Hinai-jidori eggs corresponded

to those of the Hinai-dori breed; i.e., Hinai-jidori chickens possess a Z chromosome derived from a Hinai-dori male parent. The data we obtained in the present study establishes the following: (1) eggshells are a good source for extracting maternal DNA, (2) DNA from eggshells is amplifiable by PCR, and (3) the eggs of Hinai-jidori can be distinguished from other eggs.

In conclusion, a simple and improved method for extracting DNA from eggshells has been described. This method will allow researchers to collect maternal DNA with a reduced need to capture female chickens. Furthermore, this method will contribute to discriminating the eggs of Hinai-jidori from other eggs on the market, and it will also assist in validating the labeling of Hinai-jidori eggs.

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Table1 Yields of recovered DNA from eggshells and allele size of each microsatellites (base)

Chicken	Egg No.	DNA recovery		Markers									
		Concentration (ng/μL)	Total (ng)	*ABR1003*	*ADL0250*	*ABR0241*	*ABR0311*	*ABR1004*	*ABR1013*	*ABR0633*	*ABR1005*	*ABR0089*	*ABR1007*
Hinai-jidori	1	27.3	819	161	159	98	208	216	133	261	207	216	228
	2	34.8	1044	161	159	98	208	216	133	261	207	216	228
	3	19.8	594	161	159	98	208	216	133	261	207	216	228
	4	22.1	663	161	159	98	208	216	133	261	207	216	228
	5	18.8	564	161	159	98	208	216	133	261	207	216	228
	6	33.9	1017	161	159	98	208	216	133	261	207	216	228
	7	27.0	810	161	159	98	208	216	133	261	207	216	228
	8	19.0	570	161	159	98	208	216	133	261	207	216	228
Hinai-dori	1	31.9	957	161	159	98	208	216	133	261	207	216	228
	2	43.6	1308	161	159	98	208	216	133	261	207	216	228
	3	32.1	963	161	159	98	208	216	133	261	207	216	228
	4	27.1	813	161	159	98	208	216	133	261	207	216	228
	5	22.9	687	161	159	98	208	216	133	261	207	216	228
	6	56.3	1689	161	159	98	208	216	133	261	207	216	228
	7	39.0	1170	161	159	98	208	216	133	261	207	216	228
	8	31.0	930	161	159	98	208	216	133	261	207	216	228
Rhode Island Red	1	17.5	525	155	157	130	208	218	133	271	207	216	228
	2	16.6	498	155	157	118	194	216	134	271	207	216	228
	3	17.9	537	155	157	130	194	217	134	261	207	218	228
	4	28.0	840	159	159	98	208	217	133	271	213	218	228
	5	23.0	690	148	157	118	194	216	134	261	207	216	228
	6	20.1	603	155	157	130	194	217	134	261	207	218	228
	7	19.1	573	155	157	118	194	216	134	271	207	216	228
	8	21.5	645	159	157	130	210	216	135	261	207	218	228

速　報

鶏の卵殻からのＰＣＲ増幅可能なＤＮＡ抽出手法の開発

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最近、我々は秋田県特産の比内地鶏のＤＮＡ識別手法を開発した。しかしながら、市場鶏卵（未受精卵）からのＤＮＡ抽出が困難なため、同法は比内地鶏の卵の識別に応用されていない。そこで、卵殻からのＤＮＡ抽出手法を開発した。卵殻を乳棒・乳鉢で粉砕し、０．５モルのエチレンジアミン四酢酸二水素ナトリウム（EDTA-2Na、pH8.0）溶液を用いて完全に脱灰した。その後、常法にしたがって、フェノールークロロホルム抽出およびエタノール沈殿を行い、ＤＮＡを回収した。その結果、従来法よりも５０倍効率的にＤＮＡが回収できた。同ＤＮＡを鋳型として、比内地鶏の識別に用いる１０個のマイクロサテライトマーカーのポリメラーゼ連鎖反応（ＰＣＲ）による増幅が可能だった。同マーカーにおける比内地鶏の卵の遺伝子型は、比内鶏と合致し、比内地鶏の卵は、ロードアイランドレッド種の卵と簡単に識別できた。簡便かつ効率的な、卵殻からのＤＮＡ抽出手法が確立したことにより、比内地鶏の卵のＤＮＡ識別が可能になった。