Germinal disc region: an appropriate source for obtaining maternal DNA from eggs

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Title: Germinal disc region: an appropriate source for obtaining maternal DNA from eggs

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Footnotes:

The RRL sequencing data have been deposited in the Sequence Read Archive database (www.ncbi.nlm.nih.gov/sra), and is available under accession no. SRP068548, SRP091563, SRP091591 and SRP091597.

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ABSTRACT

Eggs may serve as an alternative source for DNA extraction. The quality of DNA extracted from eggshell, whole egg liquid (WEL) and germinal disc region (GDR) was compared based on the spectrophotometric, electrophoretic, PCR and reduced-representation library sequencing (RRLS) results. Although these DNAs were all invisible on the gel and can not be measured spectrophotometrically, the GDR DNA was superior to the eggshell and WEL DNA in PCR efficiency. After the whole genome amplification (WGA) was introduced, the yield of GDR DNA was significantly increased. The obtaining DNA had overwhelming superiority over the eggshell and WEL DNA in the ratio of captured genome and the number of called SNP. The GDR DNA extraction followed by the WGA provides a method to obtain sufficient DNA from a single egg.

Keywords: egg, DNA extraction, germinal disc region, whole genome amplification, reduced-representation library sequencing

INTRODUCTION

In the poultry genetic study, blood is the most common source for DNA extraction. But, during collection of blood samples by veinpuncture, it can incur unfavorable stress to the laying hens, and for free-range domestic fowl, i.e. ducks or wild birds, it is relatively difficult to capture them. Eggs provide an alternative source because some studies confirmed that the maternal DNA can be successful isolated from whole egg liquid (WEL; Herman, 2004), yolk (Sun et al., 1998), dried debris within museum bird eggs (Lee and Prys-Jones, 2008), eggshell (Rikimaru and Takahashi, 2009), eggshell membrane (Hu and Wu, 2008), and even from fossil avian eggshell (Oskam et al., 2010). However, the egg constitution that a few of cells exist in the environment rich in proteins and lipids poses a challenge to DNA extraction (He et al., 2007).

Using the resulting DNA as a template enabled the amplification of short fragments of DNA that are less than 300 bp. However, due to low concentration and poor quality of the DNA, it is difficult to amplify fragments that are longer than 300 bp (He et al., 2007; Herman, 2004; Lee and Prys-Jones, 2008; Rikimaru and Takahashi, 2009).

The germinal disc region (GDR) is a white plaque on the surface of a yolk from which an embryo begins to develop in a fertilized egg. Previously, DNA was successfully extracted from granulosa, theca and blastoderm cells in the GDR of F2 follicles and fertilized eggs (Yao et al. 1998; Steiner et al. 2011). These studies suggest that it can be also feasible to isolate maternal DNA from the GDR of unfertilized eggs. Here, GDR DNA extraction was attempted. The quality of the GDR DNA was compared to that of the eggshell and WEL DNA based on the spectrophotometric, electrophoretic, PCR and reduced-representation library sequencing (RRLS) results.

MATERIALS AND METHODS

EGGS

Unfertilized duck eggs were used as materials from which DNA was extracted. The eggs were collected from two duck farms at Zhouzhi town, Shaanxi province of China. The egg collection and research protocol were approved by the Northwest A&F University Animal Care and Use Ethics Committee and all experiments were conducted in compliance with Canadian Council on Animal Care guidelines.

DNA EXTRACTION

Eggshell DNA was extracted according to the methods described by Rikimaru and Takahashi (2009). Starting amounts of eggshell were 40 mg and 100 mg.

Egg white and egg yolk were mixed thoroughly to form WEL. Extractions of DNA from 50 μ L, 100 μ L and 200 μ L of WEL were performed with DNeasy Blood &

Tissue Kit (QIAGEN, Valencia, CA) following manufacturer's instructions. Two hundred μL of WEL, exceeding maximum amount of starting materials in the QIAGEN protocols, was first centrifugated at 10000 rpm for 5 min. The resulting pellets were used as the starting materials. Extraction of DNA from 2 mL of WEL was conducted using the phenol–chloroform method (Sambrook and Russell, 2006). Similarly, a centrifugation process was performed before extraction. The resulting pellets from 2 mL of WEL were used as the starting material.

In the GDR protocol eggs were first stored at 4 °C for over 6 h to decrease the mobility of the yolk. The precooled egg was broken into a Petri dish. The GDR was dissociated from the surface of the yolk by cutting off the perivitelline membrane, and transferred to a 1.5 mL microcentrifuge tube with a pipette. During GDR isolation the yolk should be disturbed as little as possible. GDR DNA was extracted using DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA) following manufacturer's instructions.

WHOLE GENOME AMPLIFICATION (WGA)

WGA was conducted using a REPLI-g Mini Kit (QIAGEN, Valencia, CA) following manufacturer's instructions. DNA extracted from 100 mg of eggshell, 100 μL of WEL, and a single GDR was used as templates for WGA.

CONCENTRATION and QUALITY EVALUATION of DNA

Concentration and quality of eggshell, WEL and GDR DNA were assessed spectrophotometrically using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). In addition, concentration and integration of the DNA were subjected to electrophoretic evaluation by separating 5 μL of DNA on a 1 % agarose gel.

Three microsatellites of CAUD027, CAUD074 and CAUD039 and three fragments within the *SLCO2B1* gene were selected as targets. The rates of PCR success were

compared between GDR DNA and eggshell DNA or WEL DNA. Three microsatellites were detected using the PCR conditions and primer sequences reported by Huang et al. (2006). Three *SLCO2B1* fragments were amplified with the primers as follows: 5'-TCGCTCCCTCACTTCATC-3' and 5'-CAGGTCGGTGGTGTGCT-3' for *SLCO2B1* 5[#], 5'-GGTCCTGCTCCTTCTGCCTTGT-3' and 5'-CTGCGCCGCATTCTTCC-3' for *SLCO2B1* 9[#] and 5'-AAGCCACCCAATTAGTGC-3' and 5'-CAACACGCCAGAGGTTCT-3' for *SLCO2B1* 10[#]. All PCR was conducted using Taq MasterMix kits (CWBIO Co., Beijing, China) following manufacturer's instructions. PCR products were separated on a 2 % agarose gel, and a clear target band was identified as PCR success. The rate of PCR success was defined as the number of successful samples divided by the total number of tested samples.

REDUCED REPRESENTATION LIBRARY (RRL) CONSTRUCTION and SEQUENCING

DNA used in the RRL sequencing (RRLS) was those extracted from a single GDR, 100 µL of WEL and 100 mg of eggshell and subjected to the WGA. A total of 8 RRL that consist of 2 blood, 2 eggshell, 2 WEL, and 2 GDR RRL were constructed. In brief, DNA was first digested with 10 units *AluI* overnight at 37 °C. The digested DNA was fractionated on a 2.5 % agarose gel, and then fragments with size between 300-400 bp were sliced out of the gel and recovered with a Gel and PCR Clean-Up System (Promega, Madison, WI). Finally, the RRLs were constructed using Illumina Sample Preparation Kits following the manufacture's instruction (Illumina, San Diego, CA). Paired-end sequencing was performed on the Illumina HiSeq2500. Raw data was filtered by removing reads with adapter, the amount of N more than 10 %, and the amount of low quality bases (Q < 5) more than 50 %. The obtaining clean reads were

mapped to duck reference genome release-82 by BWA software. SNPs were called with SAMtools and filtered according to two conditions: supporting reads > 4 and Phred quality score > 20.

RESULTS AND DISCUSSION

This study aimed to establish a method of obtaining sufficient and high quality DNA from a single unfertilized egg to meet the needs of variant analysis. In this study, three egg components of eggshell, WEL and GDR were used as the starting materials of DNA extraction. The yield and quality of DNA were compared among these protocols.

Neither could eggshell, WEL and GDR DNA be evaluated spectrophotometrically with a negligible absorbance peak at 260 nm, nor could they be visualized on an agarose gel (Figure 1). This indicated that the yield of egg-derived DNA was extremely low regardless of which fractions were used. In the eggshell and WEL protocols different amounts of the starting materials were used. The DNA was consistently invisible on the gels, indicating that to increase the starting amounts had no obvious effect on DNA yield (Figure 1). On the contrary, overlarge amounts can increase cost because the amount of extraction buffers increases proportionally.

Although the yield of GDR DNA fell short of our expectation, the PCR efficiency was substantially increased, especially for the amplification of long DNA fragments. Here, three microsatellites were successfully amplified in more than 73 % of the samples (Table 1). The rates for long fragments ranged from 11 % to 47 %, which were significantly higher than the rates of PCR success in the eggshell and WEL groups (Table 1). The evident improvement may be relevant to two reasons: (1) Compared to eggshell and WEL, GDR, which consists of overlying granulose cells and the oocyte's nucleus and most organelles (Yao et al. 1998), is a region in which

maternal genetic material is mainly distributed. (2) Several studies have reported that the large amounts of proteins, lipids and cations in eggs have an adverse effect on PCR (Herman, 2004; He et al., 2007; Steiner et al., 2011). Here, the adherent egg components were avoided as much as possible during the GDR isolation. Methodologically, this operation weakened the inhibitory action of these egg components to PCR.

After WGA was introduced, concentration of GDR DNA was increased to 28.9 ± 10.3 ng/ μ L. In line with the spectrophotometric result, the whole-genome-amplified GDR DNA showed a band similar to one displayed by blood DNA (Figure 1). With the increasing of DNA yield, the rates of PCR success were increased to 43 % to 66 % for long fragments, which were significantly ($P < 0.05$) higher than those observed when original GDR DNA was used as template (Table 1). However, the improvement was not significant for the amplification of microsatellites (Table 1). The WGA did not exert a similar role on the eggshell and WEL DNA. Here, the whole-genome-amplified DNA remained invisible on the gel and could not be assessed spectrophotometrically (Figure 1). The rates of PCR success were slightly improved for microsatellites, but not for the amplification of long fragments (Table 1).

Recently, RRLS provides a powerful method for the study of genomic variants due to high throughput and low cost (Davey et al., 2011). Here, the feasibility that GDR DNA was subjected to the RRLS was assessed, and the RRLS result was compared with ones of blood, WEL and eggshell DNA. Four sources of DNA showed almost the similar ratio of Q20 bases, which indicated that there was no significant difference in the sequencing accuracy (Table 2). But, the ratio of captured genome had substantial difference between different sources of DNA as the coverage at least $1 \times$ showed (Table 2). For the blood DNA, average 12.54 % of genome was captured by the RRL

(Table 2). For the GDA DNA, the ratio was 5.44 % (Table 2). But for WEL and eggshell DNA, there were only 0.09 %-0.14 % of genome targeted by the RRL (Table 2). Correspondingly, the number of called SNP was the largest for blood DNA, and the second was the GDR DNA (Table 2). For the WEL and eggshell DNA, there were only less than 1500 SNP called in the RRLS (Table 2). The average TS/TV ratio for the GDR DNA was 2.481 which approached to the ratio of 2.3 reported in a previous SNP calling study (Kraus et al., 2011). Approximate 1.0 % of these SNP were distributed within the exons, 33.5 % of them were in the intron and the rest was located in the intergenic region, which was very similar to the distribution of SNP called in the blood DNA sequencing (Table 2). Taken together, the above results indicated that these SNP called in the GDR RRLS should result from true nucleotide polymorphisms instead of sequencing errors. Although the number of SNP called in the GDR RRLS was significantly lower than the one in the blood RRLS, the amount had overwhelming superiority over ones called in the WEL and eggshell RRLS (Table 2).

Our data indicated that purification of DNA from eggshell and WEL was not the optimal protocols. Not only did GDR DNA extraction followed by WGA overcome the limitation in DNA yield from a single unfertilized egg, but obviously improved efficiency of PCR and enable the analysis of genomic variants by the RRLS, thus providing a method to obtain sufficient and high quality of DNA from eggs.

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Table 1 Comparison of the rates of PCR success among different DNA extraction protocols

DNA-extracted Source	amount of starting materials	of methods	PCR success samples/total tested samples					
			CAUD027 (111-119 bp)	CAUD074 (109-121 bp)	CAUD039 (196-206 bp)	SLCO2B1 5# (1325 bp)	SLCO2B1 9# (1521 bp)	SLCO2B1 10# (1187 bp)
Blood	10 µL	phenol-chloroform extraction	5/5	5/5	5/5	5/5**	5/5**	5/5
ddH ₂ O	10 µL	QIAGEN kit	5/5	5/5	5/5	5/5**	5/5**	5/5
	-	-	0/3*	0/3**	0/3*	0/3	0/3	0/3
eggshell	40 mg	phenol-chloroform extraction	0/12**	0/12**	0/12**	0/12	0/12(*)	0/12**
	100 mg	phenol-chloroform extraction	0/12**	0/12**	0/12**	0/12	0/12(*)	0/12**
whole egg liquid		whole genome amplification	5/12*	2/12**	4/12*	0/12	0/12(*)	0/12**
	50 µL	QIAGEN kit	0/12**	0/12**	0/12**	0/12	0/12(*)	0/12**
	100 µL	QIAGEN kit	1/12**	1/12**	1/12**	0/12	0/12(*)	1/12**
		whole genome amplification	2/12**	3/12**	3/12**	0/12	1/12(*)	0/12**
	200 µL ¹	QIAGEN kit	0/12**	0/12**	0/12**	0/12	0/12(*)	0/12**
	2 mL ¹	phenol-chloroform extraction	0/12**	0/12**	0/12**	0/12	0/12(*)	0/12**
germinal disc region	a single germinal disc region	QIAGEN kit ²	56/71	60/71	52/71	8/71	21/71	34/71
		whole genome amplification	60/71	65/71	58/71	31/71**	33/71*	47/71*

¹ Whole egg liquid was centrifugated at 10000 rpm for 5 min before DNA extraction. The resulting pellets were used as the starting materials of DNA extraction.

² Rate of PCR success in the group was designed as the criterion. The rates of PCR success in other groups were compared with the criterion, respectively. Statistical significance was tested by *Chi-squared* test. (*) Continuity adjusted *P* = 0.0686, * Continuity adjusted *P* < 0.05, ** Continuity adjusted *P* < 0.01.

Table 2 Comparison of sequencing results of reduced-representation libraries from blood-, GDR-, WEL- and eggshell-derived genomic DNA

DNA-extracted Source ²	Clean base (bp)	Q20 (%)	Mapping rate (%)	Average depth (×)	Coverage at least 1× (%)	Coverage at least 4× (%)	Number of SNP	ts/tv ³	Exonic SNP	Intronic SNP	Intergenic SNP
Blood 1	900,640,512	95.62	74.94	9.87	12.76	4.65	226872	2.284	2157	71210	153491
Blood 2	1,019,959,776	95.02	82.78	11.36	12.32	5.17	273399	2.356	2914	88786	181677
GDR ¹ 1	917,733,888	95.33	79.86	9.93	5.84	2.35	148846	2.475	1487	49708	97651
GDR ¹ 2	448,445,952	95.06	78.95	6.02	5.03	1.92	124271	2.487	1491	41803	80977
WEL ¹ 1	695,709,792	96.00	85.97	134.84	0.09	0.03	1400	2.333	9	434	957
WEL 2	19,545,696	96.19	30.09	11.31	0.14	0.03	1889	2.143	38	469	1382
Eggshell ¹ 1	15,850,368	96.38	18.23	7.06	0.13	0.01	716	2.319	15	265	436
Eggshell ¹ 2	383,732,928	96.50	9.04	43.64	0.14	0.02	1251	2.257	12	353	886

¹ GDR = germinal disc region, WEL = whole egg liquid

² DNA is respectively extracted from GDR, 100 µL of WEL and 100 mg of eggshell, and subjected to whole genome amplification.

³ ts/tv = transition/transversion

Figure 1 Agarose gel electrophoresis shows genomic DNA extracted from duck eggs and blood. Five µL of DNAs extracted from eggshell, whole egg liquid (WEL), germinal disc region (GDR) of duck eggs and blood are separated on a 1.0 % agarose gel with ethidium bromide at 120 voltage for 30 min, and visualized by UV illumination. Lane 1: DM2000 DNA marker (CWBIO Co., Beijing, China) for size determinations; Lane 2-3: DNAs are respectively extracted from 10 µL of blood using phenol-chloroform extraction (PCE) and QIAGEN Kit (QK); Lane 4-6: DNAs are subjected to whole genome amplification (WGA); Lane 7: DNA is extracted from GDR of a single unfertilized duck egg; Lane 8-11: DNAs are respectively extracted from 50 µL, 100 µL, 200 µL and 2 mL of WEL; Lane 12-13: DNAs are respectively extracted from 40 mg and 100 mg of eggshell.

94x36mm (300 x 300 DPI)