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F-MSAP: A practical system to detect methylation in chicken genome

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Abstract **By replacing radiation with fluorescent system in the technique of methylation sensitive amplified polymorphism (MSAP) and optimizing reaction conditions, a modified technique to detect DNA methylation called F-MSAP (fluorescent labeled methylation sensitive amplified polymorphism) was developed. In the present study, cytosine methylation patterns of genomic DNA were investigated in two inbred chickens and their F_1 hybrids. Three types of methylation patterns were observed in each individual, namely fully methylated, hemi-methylated or not methylated types. The average incidence of methylation was approximately 40%. The percentage that the F_1 hybrid individual inherits the methylation for any given sites from either/both parent amounted to 95%, while the percentage of altered methylation patterns in F_1 individual was only 5%, including 14 increased and 12 decreased methylation types, demonstrating that F-MSAP was highly efficient for large-scale detection of cytosine methylation in chicken genome. Our technique can be further extended to other animals or plants with complex genome and rich in methylation polymorphism.**

Keywords: methylation sensitive amplified polymorphism, fluorescent labeled, chicken, genome, DNA methylation.

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The methylation of DNA is one of the main epigenetic modification patterns in eukaryotic cells, playing an important role in the regulation of gene expression. A lot of studies showed that DNA methylation may cause cell differentiation, embryo growth, X chromatin inactivation, genomic imprinting, disease and cancer[1−3]. Methods for detecting DNA methylation have been steadily improved along with the study of methylation. The classical method of analyzing methylation combines restriction enzyme digestion and the southern hybridization, based on the property of some restriction enzymes not cutting methylated DNA. In principle, this method can detect most methylation status of CpG islands, but it requires a large amount of DNA and high degree of methylation in the genome[4]. Bisulfites method overcomes some weaknesses in restriction method by applying sodium bisulfites to convert all unmethylated, but not methylated cytosines to uracil to facilitate the detection of methylation, requiring only small quantities of DNA. However, prior knowledge of the genomic sequence is needed[5]. Recently developed methods for detecting methylation based on microarray can satisfy high resolution requirements, but it is mainly used in the cases where special equipment is needed and the cost is high[6].

Methylation sensitive amplified polymorphism (MSAP) based on restriction enzyme and PCR amplification is more sensitive, simpler, and cheaper than other methods for investigating the whole genome methylation and does not require any prior information of nucleotide sequence[7]. Now MSAP is extensively applied in many areas to explore the associations between methylation and plant phenotypic instability under various induced conditions[8,9], the abnormality of cultured plant and cloned animals[10,11], and performances of hybrids, etc.[7,12]. The MSAP, however, adopts radioactive labeled substrate which is unsafe to human beings, and its reaction products need to be analyzed by gel electrophoresis system followed by X-ray film detection. This is a sophisticated approach since it requires frequent preparation of fresh labeled substrate during the experiment[7,12,13]. In the current study, a practical assay was established based on fluorescent system using dye labels. Equipped with GeneScan analysis software, internal lane size standard, and ABI PRIS M 377 DNA sequencer, this system was applied to investigate cytosine methylation patterns of chicken genomic DNA from two parental lines and their F_1 hybrids.

1 Materials and methods

1.1 Materials

Animals White Leghon (♂), White Plymouth Rock (♀) and their F_1 hybrids were used in the experiment to test methylation detection system. Genomic DNA was extracted from blood samples.

1.2 Adapters and primers

The adapters and primers were as described by Xu et al.[14] with some modifications. *Hpa* II-*Msp* I (H-M) adapter: 5′-GAC GAT GTC TAG AA-3′, 3′-CTA CTC AGA TCT TGC-5′; *Eco*R I (E) adapter: 5′-CTC GTA GAC TGC GTA CC-3′, 3′-CAT CTG ACG CAT GGT TAA-5′; preamplification primers: 5′-GAT GAG TCT AGA ACG GT-3′(H-M+1), 5′-GAC TGC GTA CCA ATT CA-3′(E+1); selective amplification: FAM-5′-GAT GAG TCT AGA ACG GTN N-3′ (H-M+3), 5′-GAC TGC GTA CCA ATT CAN N-3′ (E+3), NN stands for the random combinations of A, C, G, T.

1.3 F-MSAP

The F-MSAP system consists of four major parts, namely restriction reactions, ligation reactions, amplification reactions, detection reactions. Fig. 1 illustrates the principle of the process.

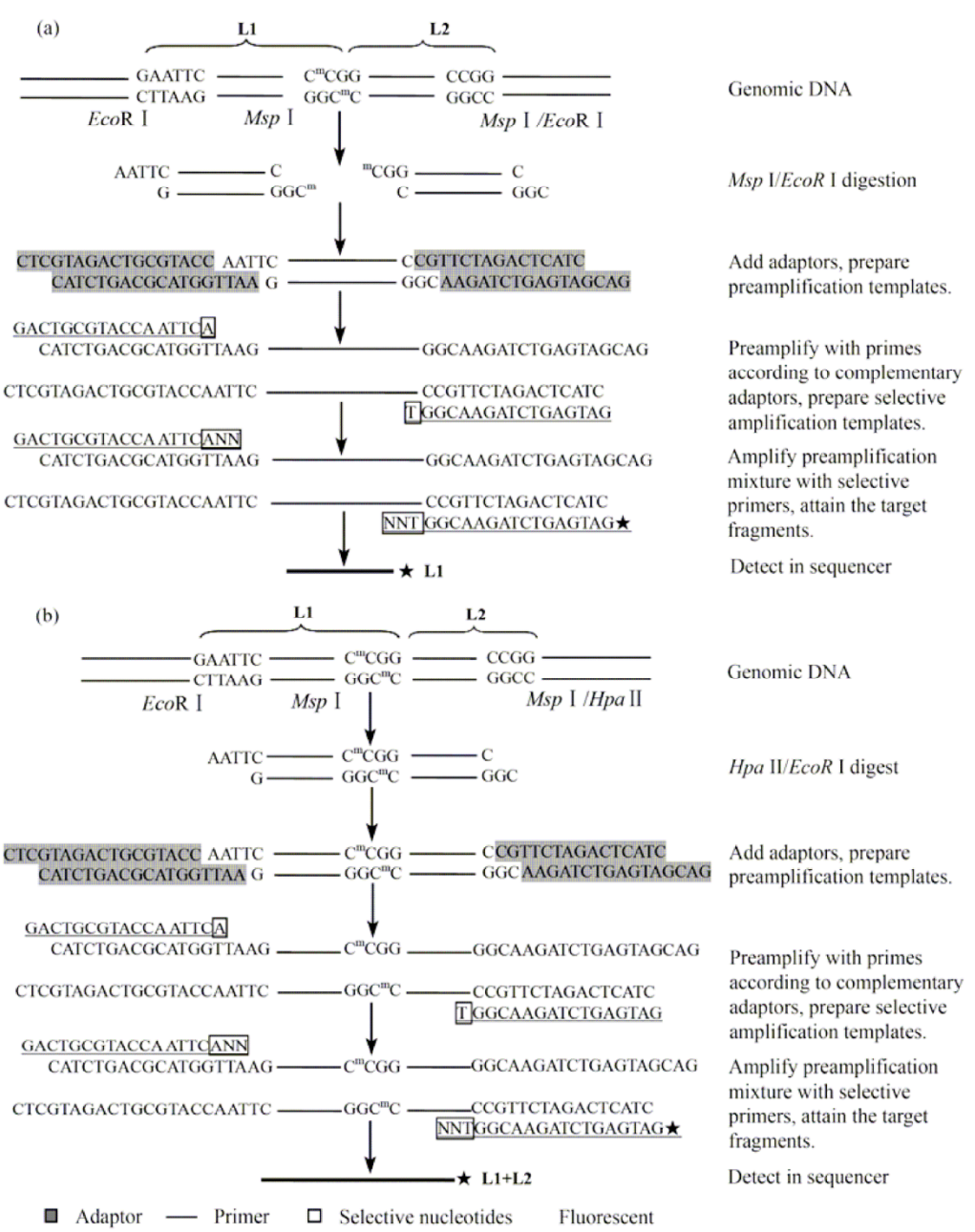

Fig. 1. Diagram of the principle of F-MSAP. (a) indicates restrictions by *Msp* I/*Eco*R I. Short fragment (L1) is generated because *Msp* I can cut $C^{5m}CGG$. (b) indicates restrictions by *Hpa* II/*Eco*R I. Long fragment (L1+L2) is generated because *Hpa* II is inactive to $C^{5m}CGG$. The genomic methylation patterns can be investigated by comparing the profiles from (a) and (b).

(i) Restriction reactions. Two digestion reactions (*Eco*R I/*Msp* I and *Eco*R I/*Hpa* II) were carried out simultaneously. In *Eco*R I/*Msp* I reaction (Fig. 1(a)), 5 μg of chicken genomic DNA was digested for 6 h in a water bath at 37℃ with 2 μL *Eco*R I/*Msp* I, 5 μ L of 10× B buffer, 1 μ L of BSA, 30 μL of sterile distilled H_2O. In *Eco*R I/*Hpa* II reaction (Fig. 1(b)), the same amount of chicken genomic DNA was digested with *Eco*R I/*Hpa* II under the same reaction conditions. Restriction enzymes *Eco*R I, *Msp* I, *Hpa* II and BSA, B buffer were purchased from Promega.

(ii) Ligation reactions. The digested DNA fragments from two digestion reactions were ligated to adaptors separately with an equal volume of ligation solution. The ligation reaction of 50 μL containing 12.5 μL of digested products, 50 pmol H-M-adapter, 5 pmol E-adapter, 3 μL of T4 DNA ligase, 5 μL of 10× buffer, 24 μL of sterile distilled H_2O was carried out at 16℃ overnight, subse-

quently, denatured for 8 min at 65℃, then cooled to room temperature, and finally stored at –20℃. T4 DNA ligase and 10× T4 DNA ligase buffer were purchased from TaKaRa.

(iii) Preamplification. The PCR reaction was performed in a final volume of 20 μL and the solution contained 0.5 μL of ligation products, 30 ng of H-M+1-primer, 30 ng of E+1-primer, 0.1 μL of *Ex Taq* polymerase, 1.6 μL of dNTP (2.5 mmol/L respectively), 1.2 μL of $MgCl_2$ (25 mmol/L), 2 μL of 10× PCR buffer, and 14.1 μL of sterile distilled H_2O. The PCR conditions were as follows: 94℃ for 5 min; 30 cycles of 94℃ for 30 s, 56℃ for 1 min, 72℃ for 1 min; finally the extension was performed at 72℃ for 7 min. *Ex Taq* polymerase, dNTP Mixture, $MgCl_2$, *Ex Taq* polymerase buffer were purchased from TaKaRa.

(iv) Selective amplification. The selective PCR reaction was performed in a 20 μL reaction volume containing 0.2 μL pre-amplification products, 30 ng H-M+3-primer, 5 ng E+1-primer, 0.1 μL *Ex Taq* polymerase, 1.6 μL dNTP (2.5 mmol/L respectively), 1.2 μL $MgCl_2$ (25 mmol/L), 2 μL 10× PCR buffer, 14.1 μL sterile distilled H_2O. The PCR conditions were as follows: 94℃ for 5 min; 13 touchdown cycles of 94℃ for 30 s, 65℃ (subsequently reduced each cycle by 0.7℃) for 30 s; 72℃ for 1 min; 23 continued cycles of 94℃ for 30 s, 56℃ for 30 s; 72℃ for 1 min; finally extension was performed at 72℃ for 7 min. *Ex Taq* polymerase, dNTP Mixture, $MgCl_2$, *Ex Taq* polymerase buffer were purchased from TaKaRa.

(v) Detection assay. 0.5 μL selective amplified products mixed 1∶1 (*v*/*v*) with loading buffer (65% deionized formamide, 10% blue dextran/25 mmol/L EDTA loading solution, 25% GeneScan^{TM}-1000ROX^{TM} size standard), heated at 95℃ for 5 min, quick-chilled on ice, the entire mixture was loaded onto a 4% denaturing Long Ranger gel, then run on the ABI PRIS M 377 DNA sequencer. The electrophoresis was performed at constant power, 3000 V, 43 mA, 108 W, for 4.5 h at 50℃. The GeneScan^{TM}-1000ROX^{TM} standard was purchased from Orbital.

2 Results

2.1 DNA methylation profiles of chicken genomic DNA generated by F-MSAP

DNA methylation profiles were developed for chicken genomic DNA by F-MSAP. Each individual genome displayed 100—150 clear bands between 50 and 2000 nt with each primer combination, as illustrated by the gel files automatically captured by sequencer and the data translated from the gel files by using Genescan3.1 software. In particular, fragments between 100 and 500 nt were highly intensive and there were a small quantity of fragments exceeding 1000 nt. The amplified products, used in F-MSAP system, were detected by silver staining method for comparison. As shown in Fig. 2, methylation profiles generated from F-MSAP system was much more informative than that from MSAP system, whose total amount of

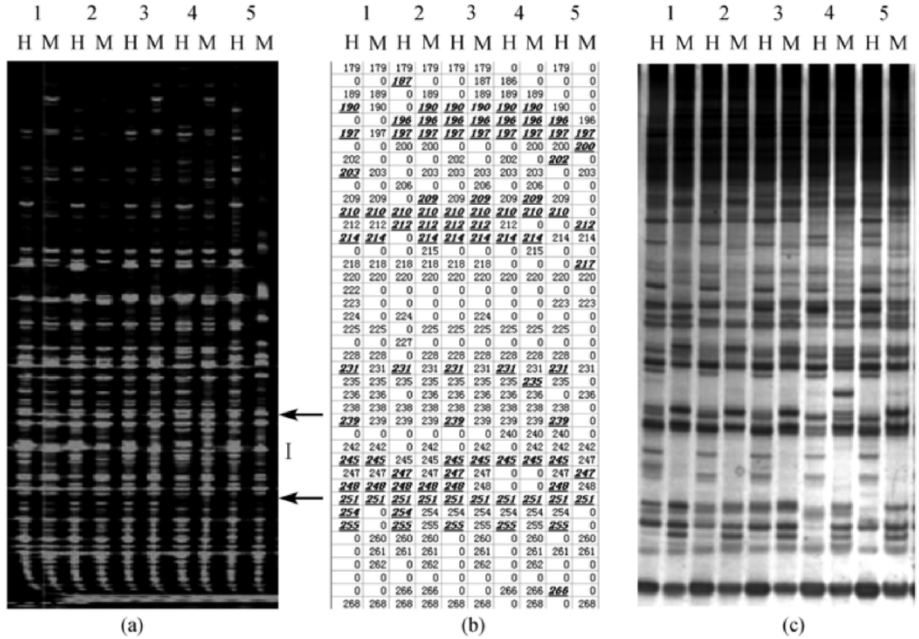

Fig. 2. DNA methylation profiles of chicken genomic DNA with the primer combination H-M+TAC/E+ACA. (a) The profile from F-MSAP; (b) portion data of I from (a) by using GeneScan Analysis software; (c) profile from MSAP using silver stain; H and M refer to digestion with *Eco*R I/*Hpa* II and *Eco*R I/*Msp* I respectively. Lanes 1—5 are the F_1 (lane 1—3), White Leghon (lane 4), and White Plymouth Rock (lane 5), respectively.

clear bands shown in Fig. 2(c) was 40% more than that in Fig. 2(a).

2.2 Cytosine methylation patterns in individual genome

Typically, the F-MSAP bands may be grouped into three types (Fig. 3): (i) bands of same length presented in both two lanes, which implies no DNA methylation events occurring in this site; (ii) unique band presented only in the H (*Hpa* II/*Eco*R I) lane, which implies a hemi-methylation at the external cytosine nucleotide in CCGG sequences; (iii) unique band presented only in the M (*Msp* I/*Eco*R I) lane, which implies a full methylation of the internal cytosine in CCGG sequences. The type (i) was the most frequently observed, representing about 60% of the total occasion, while types (ii) and (iii) accounted for about 30% and 10%, respectively.

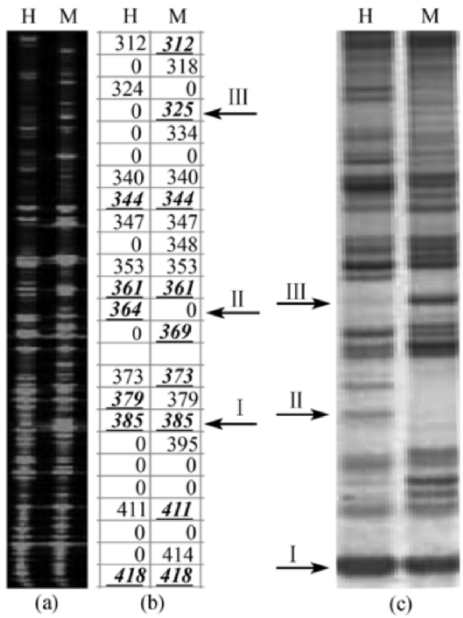

Fig. 3. Cytosine methylation patterns with the primer combination used was H-M+TAC/E+ACA. (a) The profile from F-MSAP using fluorescent dye-labeled primer; (b) part data of A by using GeneScan Analysis software; (c) profile from MSAP using silver stain. H and M refer to digestion with *Eco*R I/*Hpa* II and *Eco*R I/*Msp* I respectively. Arrow I, II and III refer to sites of no methylated, hemi-methylated, and fully methylated, respectively.

2.3 Methylation patterns among two parental lines and their F_1 hybrids

The methylation patterns of the two parental lines and their F_1 hybrid were compared. In current study, two patterns were identified. (i) Inherited pattern, the methylation patterns in F_1 hybrids were in accordance with that in both or either parental lines. In experiment, the ratio of the F_1 hybrid inheriting the methylation from both parents for any given sites was approximately 42%, and that from either parent was approximately 53%. (ii) Altered pattern, the appearance of a different methylation pattern in F_1 individuals when compared to its parental lines was considered as an alteration; it accounted for about 5% and includes 14 increased and 12 decreased methylation types. Results are listed in Table 1.

3 Discussion

3.1 Comparison between MSAP and F-MSAP

MSAP is a modified AFLP (amplified fragment length polymorphism) technique[15] to investigate cytosine methylation in genomes. In brief, isoschizomers *Hpa* II and *Msp* I, recognizing the same sequences but differing in their sensitivities to methylation of their recognition site, are used instead of *Mse* I to digest the genomic DNA, then methylation sensitive polymorphic fragments can be generated after PCR amplification with compatible adapters and primer. Theoretically the development of AFLP will certainly promote the improvement of the MSAP. Huang and Sun[16] adopted fluorescent labeling in 1999 to improve AFLP system, obtained higher differentiability and detected 10%—30% more polymorphism than the conventional system. Other experiments[17,18], also proved that fluorescent labeling system is more sensitive, safer and more practical than other detection methods. The same idea was employed in the present study to improve MSAP. The main advantages of F-MSAP over MSAP can be summarized as follows: (i) safe, radiation was completely avoided due to the use of fluorescent dye instead of radioactive labeled substrate; (ii) time efficient, autoradiography of radioactive system takes about 72 h, while there is no radiography step in F-MSAP; and (iii) highly sensitive and automatic, the tedious procedure of silver staining is avoided. Furthermore, the automated system for subsequent analysis of the data by generating exportable raw data in F-MSAP facilitated experiments requiring high resolution.

3.2 Interpretation of results from F-MSAP system

The specificities of *Hpa* II and *Msp* I are described in the REBASE database of restriction enzymes[19]. In summary, *Hpa* II and *Msp* I recognize the same sequence CCGG, but display different sensitivities to DNA methylation. The enzyme *Hpa* II is sensitive to methylation of either cytosine residue at the recognition site (CCGG), whereas its isoschizomer *Msp* I is sensitive only to methylation of the external cytosine. Therefore, methylation of the cytosine would lead to a different cleavage by these two isoschizomers and thus cause different appearances of MSAP fragments (*Eco*R I–*Eco*R I, *Eco*R I-*Hpa* II, *Eco*R I-*Msp* I, *Msp* I-*Msp* I, and *Hpa* II-*Hpa* II) in the sequencing gel loaded with the amplification products from *Eco*R I/*Msp* I and *Eco*R I/*Hpa* II digestions. The bands *Eco*R I-*Hpa* II or *Eco*R I-*Msp* I are preferentially amplified.

It is well known that the majority of CCGG sites in genome are un-methylated. The proportion of un-methylated (pattern I, Fig. 3) CCGG sites in this study was 60%,

Table 1 Methylation pattern alterations in F_1 hybrid [a)]

White Plymouth Rock		White Leghon		F_1		Type	White Plymouth Rock		White Leghon		F_1		Type
H	M	H	M	H	M		H	M	H	M	H	M	
0	0	0	0	1	1	D1	1	1	0	1	1	0	D14
1	0	0	0	1	1	D2	1	0	1	0	0	0	I1
0	0	0	1	1	1	D3	0	1	0	1	0	0	I2
1	0	1	0	1	1	D4	1	1	1	0	0	0	I3
0	1	0	1	1	1	D5	1	1	1	0	0	1	I4
0	0	1	0	1	1	D6	1	1	1	1	0	1	I5
0	1	0	0	1	1	D7	1	1	1	1	1	0	I6
0	0	1	0	0	1	D8	0	1	1	1	0	0	I7
0	0	0	0	0	1	D9	0	1	1	1	1	0	I8
1	0	0	0	0	1	D10	1	0	1	1	0	1	I9
0	0	0	0	1	0	D11	1	0	1	1	0	0	I10
0	1	0	0	1	0	D12	1	1	1	1	0	0	I11
0	1	0	1	1	0	D13	1	0	1	0	0	1	I12

a) D, Decreased methylation; I, increased methylation; 1, band is present; 0, band is absent.

and it corresponded with the result of Xu et al.[14]. However, the results for another two patterns were different from that of Xu et al. This may be due to the difference in materials. Hemi-methylation sites maybe occur more frequently at full methylation sites in chicken genome than in plants genome. In addition, technically speaking, the different labeling philosophies for the primers employed in the present study may also contribute to this difference. The primers H-M+3 instead of E+3 were labeled with fluorescence dye in the selective amplifications in F-MSAP in order to increase the precision of the occurrence of selective amplification production related to CCGG site on a denaturing gel, since the possibility for the F-MSAP system to recognize *Eco*R I-*Eco*R I fragments is almost zero. Furthermore, the automatic methylation detecting technique using DNA sequencer in F-MSAP system is capable of catching the poor signal of hemi-methylation fragments, while the autoradiography or silver staining technique in MSAP system is not under the same condition.

3.3 Recommendations

Adapters and primers proposed by both Xu et al.[14] and Xiong et al.[7] were applied. Amplification was more efficient when adopting adapters and primers for *Hpa* II- *Msp* I digest fragments from Xu et al. than from Xiong, which confirmed the effectiveness of the improvement by Xu et al. in identifying methylated DNA profiles. Additionally, the primers for selective amplification were more efficient when adding three nucleotides to the preamplification primers in our experiment on chicken genome, than adding four nucleotides to their preamplification primers in other experiments on plant genome. This phenomenon might indicate that the methylation degree and the number of CCGG sites in chicken genome are different from that in plant genome. Initially we adopted the experiment condition proposed by Xu et al.[14] in our study; however, we made some changes to pursue the best results considering the difference between chicken and plant genome. So it is recommended that when referring to related literatures for prompt initiation, one should establish his/her OWN experiment system according to his/her objective and experiment material but not just follow the related reference.

4 Conclusion

This study adopted fluorescent labeling technique to improve MSAP and established F-MSAP system. For the first time, the system was used to make an investigation of DNA methylation patterns throughout the entire chicken genome. The results clearly demonstrated that F-MSAP is highly efficient for large-scale detection of cytosine methylation and can be further extended to research on genome of other animals and plants that have complex genome and are rich in methylation polymorphism.

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