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Full Length Research Paper

Genotyping of flavin-containing mono-oxygenase 3 (FMO3) gene by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and mismatch amplification mutation assay (MAMA-PCR) in chickens

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Fishy smelling off-flavour of chicken meat is caused by a mutation (*FMO3 c.985A>T*) in exon 7 of flavin-containing mono-oxygenase 3 (FMO3) gene. Comparative analysis of the causative mutation in FMO3 gene in different chickens using two different methods was reported. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was based on general primers, whereas another employed method was the mismatch amplification mutation assay (MAMA) primers. Seventy (22.88%) of the 306 samples were genotyped using PCR-RFLP, 194 (63.40%) of the 306 samples were genotyped using MAMA-PCR and 42 (13.72%) of the 306 samples were genotyped by both of PCR-RFLP and MAMA-PCR and genotyping data were validated by DNA sequencing. The results show that the genotyping accuracy for PCR-RFLP was 90.48% in AA, 85.42% in AT and 95.35% in TT and 100% in AA, 98% in AT and 96% in TT for MAMA-PCR. This study suggests that the MAMA-PCR is more effective, accurate, easy, fast, efficient and reliable than PCR-RFLP.

Key words: FMO3 gene, chicken, mutation, PCR-RFLP, MAMA-PCR.

INTRODUCTION

Mullis et al. (1985) invented the polymerase chain reaction (PCR) method. From that time, it has become one of the most widely used methods in molecular biology (Cao et al., 2009). Flavin-containing mono-oxygenase 3 (FMO3) is a gene that is clustered on human chromosome 1 (HSA1) (Shephard et al., 1993), chicken chromosome 8, cattle chromosome 16 and pig chromosome 9 and responsible for fishy off-flavour in chicken eggs (Honkatukia et al., 2005; Kretzschmar et al., 2007, 2009; Ward et al., 2009); cow's milk (Lunden et al., 2002) and pork (Glenn et al., 2007) and also in human, which is called trimethylaminuria or 'fish-odour syndrome' (Humbert et al., 1970; Al-Waiz et al., 1987; Zhang et al., 1995; Dolphin et al., 1997; Hernandez et al., 2003), resulting from loss of function mutations. The causative mutation is an A to T SNP at nucleotide 985 of the coding sequence (*FMO3 c.985A > T*) that causes a threonine to serine substitution at amino acid 329 (Honkatukia et al., 2005) and the reported genomic *FMO3* sequence for *Gallus gallus* (GenBank accession number AJ431390.1). Although, several different methods are available to detect mutations in gene, here, we developed PCR-RFLP and MAMA-PCR to detect mutations in FMO3 gene. Our

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Abbreviations: FMO3, Flavin-containing mono-oxygenase 3; **PCR-RFLP,** polymerase chain reaction-restriction fragment length polymorphism; **MAMA,** mismatch amplification mutation assay.

Table 1. Chicken numbers for different breeds and genders.

Gender\Breed	Male capons	Male uncapons	Female	Total
BF	27	9	43	79
WL	17	66	8	91
WR	44	12	40	96
TC	12	12	12	36
NB	-	2	-	2
HB	-	-	2	2
totals	100	101	105	306

BF, Beijing Fatty Chicken; WL, White Leghorn; WR, White Rock; TC, Tibetan Chicken; NB, Nongda Brown; HB, hybrid of Beijing Fatty Chicken and White Rock.

goal was to compare two genotyping methods for the FMO3 gene in different chicken breeds.

MATERIALS AND METHODS

Collection of chicken samples

A total of 306 breast meat samples were collected from six different chicken breeds including Beijing Fatty Chicken (BF), White Leghorn (WL), White Rock (WR), Tibetan Chicken (TC), Nongda Brown (NB) and a hybrid of Beijing Fatty Chicken and White Rock (HB). All chickens were 120 days old with same feedstuff, and were bred in the China Agricultural University Poultry Farm. The meat was obtained within 2 h of slaughter and transferred to the freezer at -20°C until used. Chicken numbers are shown in Table 1.

DNA isolation from Chicken meat

Genomic DNA was extracted from meat samples. The procedure for extraction was modified from that of "Molecular Cloning" (Sambrook et al., 2002) as follows. 30 to 50 mg of meat was taken and 500 µl lysis buffer was added (10 mM Tris pH 8.0, 100 mM NaCl, 10 mM EDTA pH 8.0, 0.5% SDS) and homogenized properly with homogenizer (PRO SCIENTIFIC INC., USA). The mixture was allowed stay at room temperature for 10 min; 500 µl phenol was added and mixed for 10 min, and separated the two phases by centrifugation at 12000 g for 10 min. Transferring the supernatant to a new tube, was added 400 µL isopropanol, inverted and mixed the tube gently, and stored at -20°C for 20 min. Centrifuged at 12000 g for 10 min at room temperature, then washed the pellet with 70% ethanol (4°C) and at last dissolved the pellet DNA with 100 µL double distilled water. DNA samples were stored at 4°C for further experiments.

Methods for PCR-RFLP

Primers design and PCR amplification

For the design of primers, we used Primer premier version 5 (Integrated DNA Technologies). The primers were as follows:

The forward primer: 5'-CAGCAGCAGCAGCCAACTTA-3';
The reverse primer: 5'-GCCTCGTTGTTCTTGCTTTCG-3'.

The amplified products were 261 bp in length. The total volume for PCR was 25 µl, including 2.5 µl of 10×*Taq* PCR buffer, 0.5 µl of dNTP (10 mM), 0.5µl of each primer, 0.3µl of *Taq* DNA polymerase, 19.7 µl of distilled water and 1µl of DNA template. The reaction began with an initial denaturation at 95°C for 4mins. This was followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 63.8°C for 30 s, and extension at 72°C for 15 s. The last step was a 5 min final extension period at 72°C. The products were then analyzed by 1.5 % agarose gel electrophoresis.

Genotyping of the FMO3 gene

The chickens were genotyped for *FMO3 c.985A>T*, which was modified from those reported by Ward et al. (2009). The amplified products were then digested with the restriction endonuclease *Bsr* I (Fermentas). The reaction volume was 10 µl, including 1.0 µl of 10×NE Buffer, 0.3 µl of *Bsr* I, 2.0 µL of PCR product and 6.7 µl of distilled water. A drop of mineral oil was added to each tube to prevent evaporation. The 10 µl reactions were incubated for 4 h at 65°C. The digested products were then electrophorized by 2.5% agarose gel to visualize the differences in fragment length.

Methods for MAMA-PCR

Design of mismatch primer

According to the reported genomic *FMO3* sequence for *Gallus gallus* (GenBank accession number AJ431390.1), we created a single nucleotide mismatch near the 3′ end to enhance the 3′ mismatch effect. The mismatch amplification mutation assay (MAMA) primers for genotyping were as follows: the normal primer for wild-type (A): 5'-CGATGCGGTTATCTTTGCCA-3'; the mismatch primer for mutant type (T): 5'-CGATGCGGTTATCTTTGCCT-3'; the common reverse primer: 5'- GCTGCCCTCATTTTTAGTTGGAA-3'. The normal primer and the reverse primer can generate a short PCR product from the wild type gene, but fail to amplify the mutated gene, while the mismatch primer and reverse primer makes the opposite result.

PCR amplification

The amplified products were 306 bp in length. The total volume for PCR was 25 µl, including 2.5 µl of 10×*Taq* PCR buffer, 0.5 µl of dNTP (10 mM), 0.5 µl of normal primer (A) or mismatch primer (T), 0.5 µl of common reverse primer, 0.3 µl of *Taq* DNA polymerase, 19.7 µl of distilled water and 1 µl of DNA template. The reaction began with an initial denaturation at 95°C for 5 min. This was followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 25 s. The last step was a 5min final extension period at 72°C. The products were then

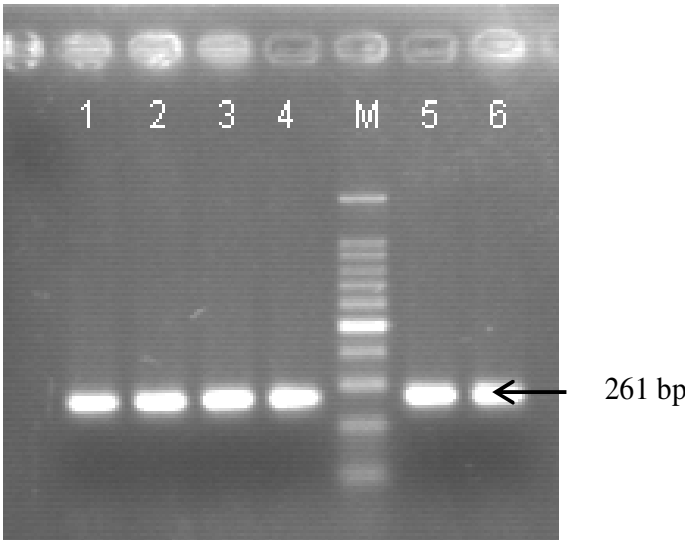

Figure 1. PCR amplification of the *FMO3 c.985A > T* by PCR-RFLP. M, Marker; lanes 1-6: 261 bp.

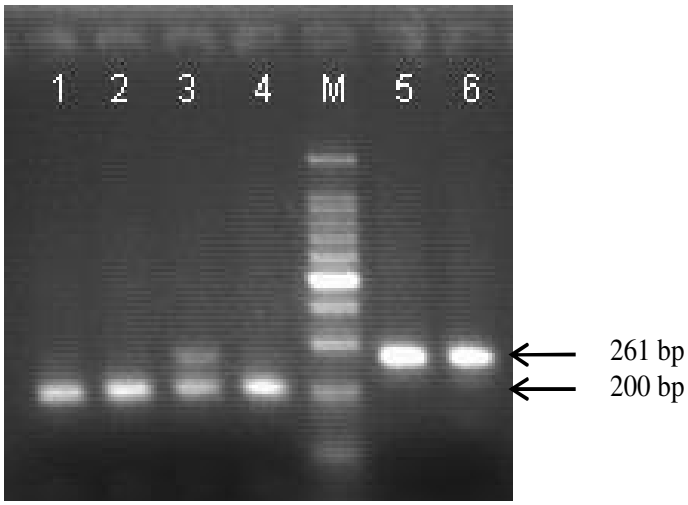

Figure 2. Genotyping of the FMO3 c.985A> T by PCR-RFLP. Lanes1,2 and 4 : 200 bp and AA genotype; lane 3: 200 and 261 bp and AT genotype; lanes 5 and 6: 261 bp and TT genotype.

analyzed by 1.5% agarose gel electrophoresis.

RESULTS

Genotyping of chicken *FMO3*

PCR-RFLP method

A summary of the PCR amplification and genotyping of *FMO3 c.985A>T* in different chickens are presented in Figures 1 and 2. In Figure 1, we used a total of 6 samples. All samples were 261 bp in length. M (Marker) was 100 bp DNA ladder.

In Figure 2, we used the same 6 samples. According to PCR band, sample 1, 2 and 4 showed 200 bp and was AA genotype. The sample 3 showed 200 and 261 bp and was AT genotype. On the other hand, the sample 5 and 6 showed 261 bp and was TT genotype. By this process, we genotyped 112 chicken samples and the genotyping data was confirmed by DNA sequencing. M (Marker) was 100 bp DNA ladder.

MAMA-PCR method

A summary of the genotyping of *FMO3 c.985A>T* in different chickens are presented in Figure 3 and we also used the same 6 samples. The left side 1-6 numbers were representative of wild-type primer or normal primer (5' CGATGCGGTTATCTTTGCCA 3') and right side 1-6 numbers were for mutant primer (5' CGATGCGGTTATCTTTGCCT 3'). Here, number 1-4 was AA chicken. So, it showed band using wild-type primer and did not show incase of mutant primer. Meanwhile, number 5 and 6 was TT chicken. So, it showed band using mutant primer, whereas did not show incase of wild-type primer. By this process, we genotyped 236 chickens and the genotyping data were confirmation of DNA sequencing. M (Marker) was DM2000 DNA ladder.

Nucleotide sequence of the *FMO3* gene

Figure 4 describes the nucleotide sequencing and also shows the design for the primer.

DNA sequencing, genotyping

In this study, the genomic DNA was sequenced using PCR products and sent to Beijing Sunbiotech Co., Ltd., Beijing, China for sequencing in both directions. From all samples, we sequenced 7 samples. From these 6 samples we sequenced 4 samples (sample no. 2, 3, 5 and 6) and according to result we got AA for 2 samples (sample no. 2 and 3) and TT for 2 samples (sample no. 5 and 6). Here, we showed only 3 samples (sample no. 2, 3 and 6). The sequencing results showed 3' end of *FMO3* gene base for homozygous A allele (wild-type genotype AA, sample no. 2 and 3) and base for homozygous T allele (mutant type genotype TT, sample no. 5 and 6).

Comparative analysis of *FMO3 c.985A>T* genotyping in different chicken populations

A summary of the genotypic frequencies of *FMO3 c.985A>T* in different chicken populations is presented in Table 2.

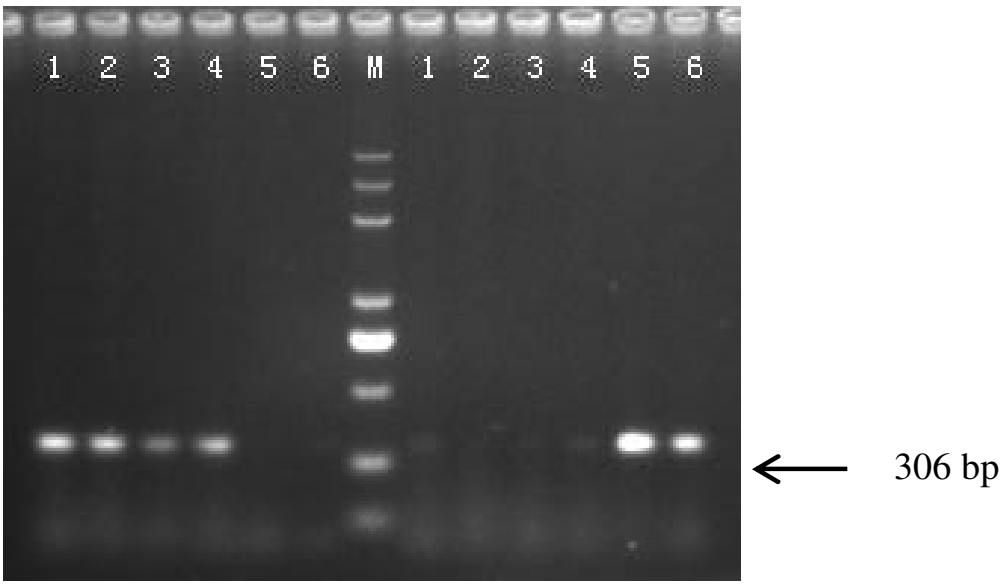

Figure 3. Genotyping of the *FMO3 c.985A> T* by MAMA-PCR. Left side: Lanes 1-6: Wild-type primer or normal primer; right side: Lanes 1-6: Mutant primer; M, marker (2000).

CTCGATGCCGTTATCTTTCCC[A/T]CTGGTTACTCTCACTCCTTTCCTTTCATG
GAGGATGAATCCATCATCGAAACCAAGAACAACCAGGCTACCCTCTACAAAT
GCATTGTTCCTCCTAAGTTGGAGAAGCCAACCATGGCAGTCATTGGGTTGGT
CCAGTCCTTTGGATCCGCCATCCCGACAGCAGATGTCCAGTGCCGCTGGGC
*AGTCAAGGTGTTTCAGG*GTAGGTGAATGAGCTAAGGTCCCTAAATGGGA
AGTAGCATTTCCCATCAGCAATTACACAGATTCCAACTAAAAATGAGGG
CAGCACATGAGTGCTGGAAATACCAGCAACAGGTTCTCCCCGGGATGG
GCTGCCTTGTGCCATGGAGAATAGGTCACTTATCAACCAGCCGCAGATG
TCAGTGAGGTCCCTGCGGTGAGGCAGCTATCTGGGCATCGCCACACATC
CTAAAGGACAGGGGGATCTGTTTTATCGCAACCCTGATGCCCAAGTTTG
ATCCAAGCTCAAGGGAAGAGAAGTGCCCAAGGATTCAGCTGACCCCTA
CAGACCCCATCTGCTGCAGGAGGGATCTCCTGGGCATCATCACCCTTTG
CCGACACTCTTCCTGTTCTTTCCAGGG

Figure 4. Nucleotide sequence of the *FMO3* gene fragment (bases exon mRNA 1013-1237 accession number: AJ431390.1). The exon is in italics and intron is in normal type. Primers are shown shadowed. For the design of mismatch primer we created a single nucleotide mismatch near the 3′ end and the A/T 985 polymorphism causing the T329S mutation is in square bracket.

Table 2. Genotyping of *FMO3 c.985A>T* in different chicken populations.

Genotype	RFLP	MAMA	**By both of RFLP and/or MAMA	*Genotyping accuracy (%) (RFLP)	*Genotyping accuracy (%) (MAMA)
AA	04	148	17(22)	90.48	100
AT	32	27	16(13)	85.42	98
TT	34	19	9(7)	95.35	96
Total	70	194	42(42)	90.42	98

*Genotyping accuracy was validated by DNA sequencing;** Same 42 samples were genotyped by both of RFLP and MAMA. But we got different genotyping results. RFLP, Restriction fragment length polymorphism; MAMA, mismatched amplification mutant assay.

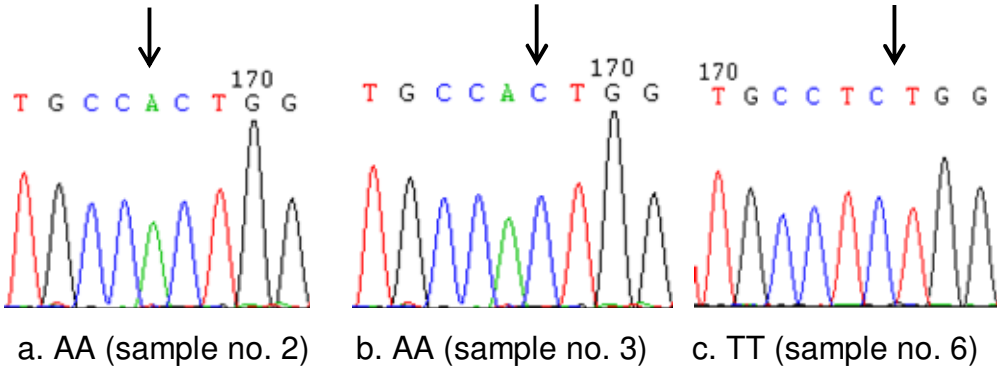

Figure 5. Sequencing identification of chicken *FMO3* genotypes.

DISCUSSION

This stuyd describes a robust PCR-RFLP genotyping protocol and a MAMA-PCR protocol for chicken *FMO3* gene that causes the T329S mutation in the coded protein. After using MAMA-PCR to detect the *FMO3* genotype, we sequenced some results from it to make sure the technology is valid way.

The comparative analysis of *FMO3 c.985A>T* genotyping is shown in Table 2. Representative gel for the *FMO3 c.985A > T* PCR-RFLP is shown in Figures 1 and 2. Genotyping of *FMO3 c.985A>T* for MAMA-PCR is shown in Figure 3. The DNA sequencing result is shown in Figure 5.

The PCR-RFLP method is a widely used method for genotyping of gene (Hao et al., 2007; Jauk et al., 2003; Koeken et al., 2002; Sironi et al., 2010; Wang et al., 2010).

For this method, there are two steps that should be done, i) PCR amplification, ii) Digestion of products with endonuclease enzyme. In this study, the PCR amplification result was excellent (Figure 1). But in case of genotyping, the enzymatic digestion result did not work out properly. Here, the endonuclease *Bsr* I digestion results were, 200 bp fragment for AA genotype, 261 bp for TT genotype and 200 and 261 bp for AT genotype. According to digestion result, we got AA for 21 samples, AT for 48 samples and TT for 43 samples of total 112 samples. Total 42 samples (17 for AA, 16 for AT and 9 for TT) of 112 were also genotyped by MAMA-PCR and the obtained results were 22 for AA, 13 for AT and 7 for TT. According to sequencing result, sample no. 3 is AA (Figure 5), but according to *Bsr* I digestion, we got an AT (Figure 2). This method is more time consuming, more expensive and difficult to operate. The result of this method was also not accurate. The genotyping accuracy for this method was 90.48% in AA, 85.42% in AT and 95.35% in TT. Although, there are some other factors, such as, concentration and quality of DNA, annealing temperature, quality of restriction enzyme, incubation temperature, all are responsible for the genotyping of the gene.

On the other hand, in MAMA-PCR, the only step amplification was required. The result was worked out properly and accurately. Here, the mismatch results were, the samples that showed band using wild-type primer and did not show using mutant primer were AA genotypes, the samples that showed band using mutant primer and did not show using wild-type primer were TT genotypes and the samples that showed band using both types of primer were AT genotypes. According to the amplification result, we got AA for 170 samples, AT for 40 samples and TT for 26 samples. According to sequencing result, sample no. 3 is AA (Figure 5), also according to MAMA-PCR; we got an AA (Figure 3). This method is time saving, less expensive, easy to operate and saves costs for the experiment. The result by this method was more accurate and proper. The genotyping accuracy for this method was 100% in AA, 98% in AT and 96% in TT.

MAMA-PCR, also known as mismatch amplification mutation assay, amplification-resistant mutation system (amplification-refractory mutation system, ARMS), allele specific PCR (ASPCR), the methods Taq DNA polymerase lacks 3'→5' exonuclease activity, the sequence containing SNPs mismatched primers, and SNPs of the base on the primer (upstream or downstream primer may be) of the 3' end. This mismatch is the most critical step in PCR. In the primer 3' end, if there are two bases that do not match, even if the combination of primers and templates, it cannot be extended; and if good control of PCR amplification conditions, primers, only one base mismatch, PCR is out well. Of course, annealing temperature and Mg^{2+} concentration is also important to adjust the links, such as the lack of amplified bands, try to increase the annealing temperature and follow the appropriate Mg^{2+} concentration. MAMA-PCR can rapidly detect a large number of species known to SNP.

This PCR method not only has the technology, it is more efficient, fast, easy, cheap and testing a large number of advantages than other methods. The limitation of MAMA-PCR can only be used in the known SNP sites. Also, as in the primer 3' end of the introduction of these principles by the mismatch of the different bases, we can artificially create a new restriction enzyme sites, and with

the RFLP technologies, the same can detect SNP sites.

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