Animal Science Papers and Reports vol. 28 (2010) no. 2, 161-170
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Sensitivity and specificity of high-resolution melting analysis in screening unknown SNPs and genotyping a known mutation*

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(Received August 17, 2009; accepted April 16, 2010)

High-resolution melting analysis (HRMA) was used to screen potential SNPs in the exons of chicken *CAPN*1 (μ-calpain/large subunit) gene. A total of 312 DNA samples from Beijing-you chickens were used for detection. Twelve pairs of primer were designed to amplify twelve different exons and SNPs were detected in five of them. HRMA was also compared with PCR-SSCP analysis for genotyping of a known SNP site in the chicken adipocyte fatty acid binding protein gene (*A-FABP*). Amplicons of 275-bp fragment, bracketing the polymorphic site, were grouped by PCR-SSCP into three genotypes designated as CC, TT and CT. Small amplicons (56 bp) within the 275-bp fragments were designed to maximize the *T*m difference between homozygotes and to genotype all possible three genotypes after a single melting analysis successfully. Results from different methods were cross-validated and sequencing results from randomly selected heterozygotes and homozygotes confirmed the specificity of HRM technique. The full consistency proved that HRMA was a useful tool for rapid, close-tube genotyping of polymorphic sites. It has great potential for SNPs detection and scanning especially on a large scale.

KEY WORDS: *A-FABP / CAPN*1 / chicken / HRMA / SSCP analysis

*Supported by the grant No. 2008AA101009 and 2006AA10Z1D8 from National High-tech R&D Program and 2007KLAN003 from State Key Laboratory of Animal Nutrition
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In marker-assisted breeding programmes, genetic alterations at functional genes need to be identified before the association of polymorphisms with animal performance is analysed. In many cases, a large number of individuals in a number of populations with diversified genetic backgrounds need to be genotyped before a positive correlation can be established between a *locus* and a trait of interest. Single strand conformation polymorphism (SSCP) analysis is a mutation detection method widely used [Sun *et al*. 2008, Wang *et al*. 2008, Zhang *et al*. 2009]. For example, the distribution of different genotypes and alleles at two SSCP *loci* in chicken tyrosinase gene was studied in a total of 424 individuals coming from three Chinese chicken breeds and an imported Hisex population [Zhang *et al*. 2008]. Ye *et al*. [2007] analysed the distribution of different genotypes at three PCR-SSCP *loci* detected in the chicken adipocyte fatty acid binding protein gene (*A-FABP*) and their relationships with fat-related traits in six Chinese local chicken breeds on a total of 728 individuals. SSCP analysis has considerable sensitivities and does not require intensive labour or sophisticated instruments to perform. But when a targeted gene is to be screened for potential SNPs or a known SNP is required to be genotyped on a large scale, it is time-consuming and laborious.

High-resolution melting analysis (HRMA), introduced in 2002 with growing popularity, is a powerful mutation-screening tool [Cho *et al*. 2008, Jones *et al*. 2008, Muleo *et al*. 2009]. It is a rapid, close-tube method to identify heteroduplex PCR products [Reed and Wittwer 2004]. Unlike conventional melting curve analysis, HRMA with saturating DNA dye (LCGreen I, Idaho Technology, Salt Lake City, USA) detects sequence variants within targeted region without the use of fluorescently labeled probes or primers and is especially useful for rapid genotyping. To evaluate the sensitivity and specificity of HRMA for screening unknown SNPs and genotyping known *loci,* we selected chicken *CAPN*1 (μ-calpain/large subunit) gene as the target gene to screen potential SNPs in the exons. A known SNP in chicken *A-FABP* gene was also selected as the target and 312 Beijing-you chickens were genotyped by SSCP analysis and by HRMA. Sequence alterations were detected by comparing the PCR product melting profiles or electrophoretic pattern after SSCP analysis with known homozygous samples and results were validated by sequencing.

Material and methods

Animals and sampling

Venous blood samples of 312 Beijing-you chickens were obtained from the Institute of Animal Science, Chinese Academy of Agricultural Sciences. Coagulation was prevented with citrate and samples were stored at -20°C until use. Genomic DNA was extracted from thawed blood using the saturated salt method [Sambrook and Russell 2001] and stored at -20°C.

High-resolution melting analysis (HRMA)

Primers used for screening single nucleotide polymorphisms(SNPs) in chicken *CAPN*1 gene were designed by Primer 5.0 and synthesized by Ubiogene Engineering Co., Ltd., Beijing, China (Tab. 1). PCR was performed in a volume of 10 μL consisting of about 40 ng of genomic DNA, 0.5 μM each of forward and reverse primers, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 2 units of *Taq* DNA polymerase (PROMEGA, Madison, WI, USA), 10 μM LC Green™ I (IDAHO TECHNOLOGY Inc. USA) with 10 μL mineral oil pipetted into each well on the 96-well PCR plate. PCR amplification was performed on a thermal block cycler (BioRad c1000, USA). Denaturing (4 min at 94°C) was followed by 32 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C, with final 10 min extension incubation at 72°C. After the amplification protocol, an extra denaturing step was applied (94°C for 30 s) followed by rapid cooling to 25°C to allow forming heteroduplexes. The amplified samples were melted from 40 to 95°C at 0.3°C/s in the high-resolution melting device (HR-1; IDAHO TECHNOLOGY Inc. USA). High resolution melting data were acquired and analysed using the accompanying HR-1 software (IDAHO TECHNOLOGY Inc.). In general, melting curves were normalized (temperature ranges on each side of the melting transition were chosen and the data points for a given sample were scaled between 0 and 100% fluorescent intensity). Temperature was adjusted by superimposing the temperature axis of each curve over the same temperature range. This aided in the differentiation between homozygotes and heterozygotes by emphasizing curve shape in conjunction with melting temperature.

Table 1. Information of primers used for screening SNPs in chicken *CAPN*1 gene

Primer		Sequence	Amplicon
calapin1-1	F:	5'-CGCAGCTATGATGCCCTTTG-3'	271 bp
	R:	5'-CATGAGCCCAGCACTTACCG-3'	
calapin1-4	F:	5'-GATCTGGCAGTTTGGTGAGTGGG-3'	149 bp
	R:	5'- GTGGTCGGACTCACTTGGCGTAA-3'	
calapin1-5	F:	5'-AGGCTGAACGGCTGCTACGAGTC-3'	198 bp
	R:	5'-CTCTGTGAAGAGCAGCGGAACACT-3'	
calapin1-9	F:	5'- CCACAGGATGTCTTTCCGGGACT-3'	191 bp
	R:	5'- CAAGAGCGGAGATGCGGGAGGTA-3'	
calapin1-10	F:	5'- CCAGGTGTATCATCCGTGTCTT-3'	242 bp
	R:	5'-GAGCCAGTCTCAAGGAAGTAGCATT-3'	

PCR-SSCP analysis

The SNP identified at the chicken *A-FABP* gene was first reported by Ye *et al*. [2007]. Primers were designed from the known chicken *A-FABP* sequence (Genbank accession AF432507) to generate a 275-bp amplicon corresponding to bases 14 to 288 in the published sequence. The primers were 5'-actgctacctggcctgac-3' (forward)

and 5'-ggaatgtgacaacgctaa-3' (reverse). It is located in the first exon of the gene and leads to a synonymous codon change from TTC to TTT. In Beijing-you chickens three genotypes, designated CC, CT and TT, were detected at this *locus*.

PCR was conducted as described above without DNA dye in the reaction system. Specific products of the expected size were analysed with SSCP. Two μL of the PCR product were diluted with 5μL of a solution containing 98% de-ionized formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol and 10% glycerol. The mixture was denatured at 98°C for 10 min, cooled in ice for at least 5 min and loaded on a non-denaturing 12% acrylamide:bis-acrylamide (29:1) gel. Electrophoresis was performed in 1×Tris Borate (pH 8.0) EDTA buffer at 10 volts/cm at 4°C overnight. DNA was detected by silver staining.

Genotyping with small amplicons

Small amplicons were designed to distinguish three different genotypes at the polymorphic site within the *A-FABP* gene by HRM assay. The forward primer (5'-cacctggaagctcctttctagtg-3') consisted of 23 bp, corresponding to bases 55 to 77, whilst the reverse primer (5'-cacccagctctttcatatagtcctc-3') consisted of 25 bp corresponding to bases 86 to 110. The 3' nucleotide of the reverse primer was just next to the SNP located at position 85 of the known chicken *A-FABP* sequence (Genbank accession AF432507); expected length of the amplicon was 56 bp. PCR reaction mixture was the same as described for HRMA. After a denaturing step at 94°C for 4 min, 32 cycles of 10 s at 94°C, 10 s at 58°C, and 10 s at 72°C were applied, terminating with a final 10 min extension incubation at 72°C. High resolution melting data were analysed with HR-1 software by fluorescence normalization and temperature overlay as already described. Homozygous genotypes were grouped on the basis of two positive fragments amplified from DNA samples with known genotypes at *A-FABP* included in each plate. A heterozygous sample was produced by mixing (1:1) two amplicons of different homozygous samples of known genotypes. Derivative plots were visualized as the negative derivative of the fluorescence relative to the temperature (-dF/dT) *versus* temperature. These steps allowed visual comparison of melting curve shapes as described by Cecily *et al*. [2004].

Results and discussion

Screening of SNPs with HRMA

Differences in melting curve shape that correlated to genotype were revealed by HRMA. Shown in Figure 1 are melting curves from homozygous and heterozygous PCR products overlaid at high temperature to visually aid comparison. Heterozygotes could always be easily identified by a change in melting curve shape. However, different homozygotes were not distinguishable from each other. Homozygous samples had sharper melting transitions, whereas heterozygotes showed broader transitions because of the duplexes formed heterogeneously.

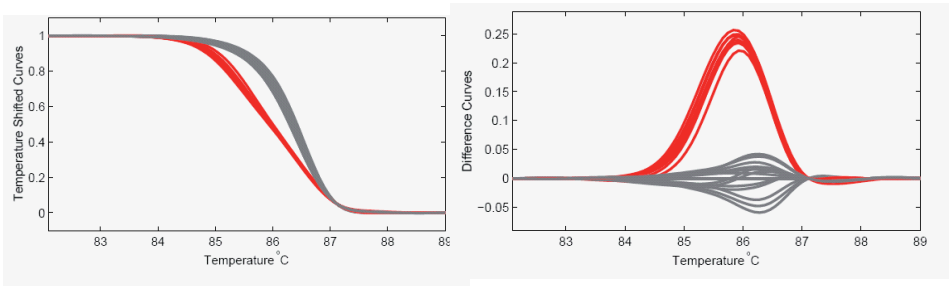

Fig. 1. Genotypes grouped by homozygotes and heterozygotes after HRMA (calpain1-10). The grey curves represent the homozygous genotypes while the red curves represent the heterozygous genotype.

Genotyping of SNP at *A-FABP* gene by PCR-SSCP analysis

PCR products from the chicken *A-FABP* gene were specifically amplified. Amplicons were obtained of the expected length of 275 bp (not shown). After SSCP analysis, three distinguishable PCR-SSCP patterns were obtained (designated CC, TT and CT (Fig. 2) in Beijing-you chickens. A total of 312 PCR products were genotyped, out of which 157 samples (50.3%) being recognized as heterozygotes. Homozygous TT and CC accounted for 26.9% (84) and 22.8% (71), respectively.

Fig. 2. Three genotypes and their designations in the chicken *A-FABP* gene, as detected by PCR-SSCP analysis.

Genotyping with small amplicons

Small amplicons were designed to separate heterozygotes and different homozygotes from each other. Results of HRMA for the 56-bps products in the chicken *A-FABP* gene are shown in Figure 3. Heterozygotes can easily be identified by the double peaks in melting curve shape, while the C/G and A/T homozygotes having similarly shaped curves (usually with a single peak) can be distinguished by a shift in melting temperature (*T*m) with the *T*m of the C/G homozygotes approximating 1°C higher than that of the A/T homozygotes (79.3°C and 78.6°C, respectively). Grouped according to the positive controls of known sequences, results from small amplicons by HRMA agreed perfectly well with that of SSCP analysis with three genotypes recognized right after the melting analysis. The complete accordance suggests that both methods provided results with excellent specificity and repetitiveness.

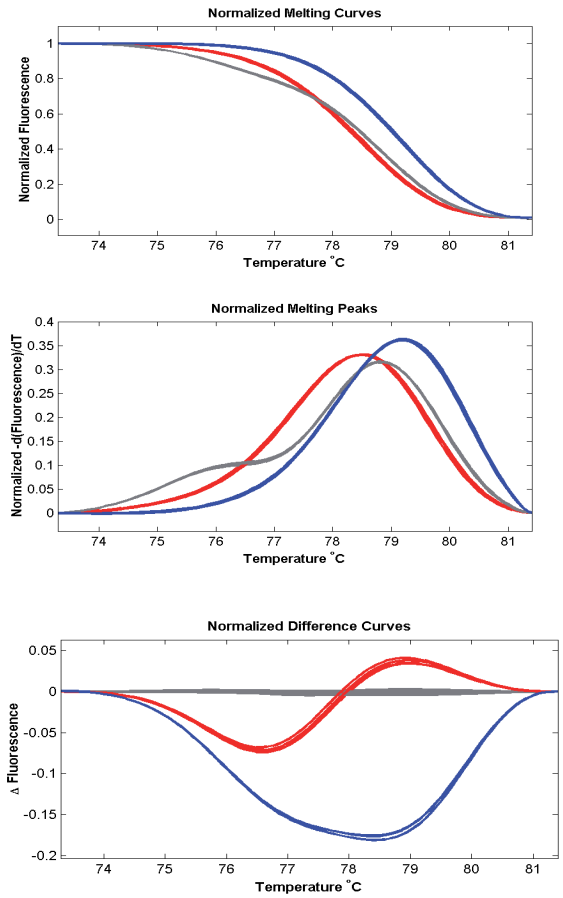

Fig. 3. Three genotypes of the chicken *A-FABP* gene grouped by HRM analysis. The grey curves represent the heterozygous genotype (CT) while the blue and red curves represent the homozygous genotypes (CC and TT, respectively).

Validation of specificity

A total of 12 pairs of primer were designed to screen potential SNPs in the exons of chicken *CAPN*1 gene with HRMA. Heterozygotes were detected in 5 amplified fragments (Tab. 1). Sequencing, which provides simultaneous genotyping and scanning is always considered to be the golden standard for SNP validation. We randomly selected 4 heterozygotes for each primer to be sequenced (ABI Prism 3100 Genetic Analyzer, Applied Biosystems). Results showed that double peaks did appear in the sequencing chromatograms for each heterozygote (Fig. 4), demonstrating that HRMA was effective in detecting unknown SNPs in the target gene (Tab. 2).

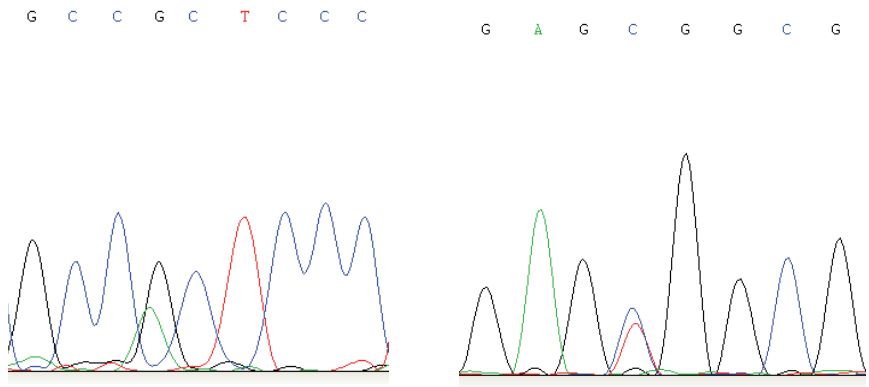

Fig. 4. Sequencing chromatograms for heterozygotes amplified by primer calpain1-10.

Table 2. SNPs detected in chicken *CAPN*1 gene by HRM assay and validated by sequencing

Location of SNP in *CAPN*1 gene (Genbank NM_205303)	Amplified by primer	Codon and AA changed	
		from	to
exon1 (142)	calapin1-1	UAU(Tyr)	UAC(Tyr)
exon4 (499)	calapin1-4	GAU(Asp)	GAC(Asp)
exon5 (676)	calapin1-5	GGC(Gly)	GGU(Gly)
exon9 (1146)	calapin1-9	CGA(Arg)	CAA(Gln)
exon10 (1304)	calapin1-10	CGG(Arg)	UGG(Trp)

As for the known SNP in chicken *A-FABP* gene, we also randomly selected 47 homozygotes (22 and 25 for TT and CC, respectively) to be sequenced for verification. There were no false positives in a total of 47 cases. Homozygotes were identical with the published variants, and the specificity was 100%.

There are many ways to genotype SNPs [Chou *et al*. 2005], including denaturing high performance liquid chromatography [Liu *et al*. 1998], denaturing gradient gel electrophoresis [Kirk *et al*. 2002] and high-density oligonucleotide arrays [Kristensen *et al*. 2001]. SSCP analysis, one of the most commonly used for SNP detection, detects sequence variations using conformation sensitive gel electrophoresis [Ganguly *et al*. 2002, Kunhareang *et al*. 2008, Jing *et al*. 2008, Hu *et al*. 2009]. It is not technically complex and is at low cost to perform. However, it requires an extra over-night past-amplification electrophoretic step and cannot be automated for high-throughput analysis. This limits its applicability when many samples need to be genotyped for a known *locus* or scanned for potential polymorphic *loci* in targeted genes. In contrast, HRMA is a good candidate to detect sequence variants on a large scale. We used LCGreen I, the heteroduplex-detecting dye, to screen unknown SNPs in chicken *CAPN*1 gene and to genotype a known SNP in the chicken *A-FABP* gene with HRMA. Unlike SYBR Green I which can only be used in limited concentrations,

LCGreen™ I saturates the products of PCR without inhibiting amplification and does not redistribute as the amplicon melts, which allows closed-tube genotyping analysis to be performed just after amplification and without conjunction with fluorescently labeled probes [Lay and Wittwer 1997, Crockett and Wittwer 2001], allele-specific PCR [Germer *et al*. 2000], or real-time PCR instruments.

Our results showed that HRMA was very sensitive to detect potential SNPs in fragments under 300 bp. The specificity of this method was also verified by a comparison with PCR-SSCP analysis. In the present study, a total of 312 samples were genotyped by PCR-SSCP analysis and cross-validated by HRMA. When the LightCycler was used, 96 samples could be analysed simultaneously, with continuous acquisition of fluorescence from 95°C to 40°C within 5 min. Once the PCR was conducted, all the samples could be genotyped in 20 min, which was much time-saving than with SSCP. Theoretically, the *T*m difference between two homozygotes, arising from transition of A/T to C/G could be detected by HRMA, which had the sensitivity to differentiate fragments with a *T*m difference of 0.3°C. However, we failed to separate two 275-bp homozygotes at the *locus* studied in chicken *A-FABP* gene (not shown). We interprete it as the result of decreasing sensitivity with the increasing length of PCR fragments.

In this study, we designed small amplicons to genotype three genotypes after PCR amplification. Since the *T*m differences among genotypes increase with the decrease of amplicon size, the amplicons were made as short as possible (56 bp) and the 3' end of each primer was placed very close to the SNP to maximize differentiation. It was shown that small amplicons, generated with PCR primers bracketing the variable *locus*, could distinguish all three genotypes easily. We conclude it to be an effective method for genotyping of known SNPs on a large scale.

When HRMA was introduced, all test and control DNA samples were recommended to be prepared in the same way and added into PCR at the same concentration for best results [Gudrun *et al*. 2007]. In this study, however, it was inconvenient to quantify more than 300 DNA samples. We simplified the procedure by adding different volumes of water to dissolve DNA according to the amount of white flocculent precipitate obtained after ethanol precipitation by experience. Although an average of 1-4 samples in each 96-well plate were discarded because of aberrant fluorescent intensity caused by too high or low template concentration, good results could still be obtained from these crude DNA samples without quantification. We concluded that if the PCR amplification was specific enough and optimized without undesired side reactions, the limited variation of initial DNA concentration could be ignored.

We conclude that HRMA, using short PCR products for genotyping, is a rapid, closed-tube method, sensitive and specific in genotyping known and detecting unknown SNPs. Different melting patterns correlate with different genotypes after a single melting analysis. It provides a convenient way to detect SNP mutations in a targeted gene without performing full fragment sequencing. Its simple handling and greatly increased throughput imply its further application in SNPs detection and scanning.

Acknowledgements*. The authors wish to thank W. Bruce Currie of Cornell University, New York, USA for language suggestions on the manuscript.*

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M.H. Ye , J.L. Chen , G.P. Zhao, M.Q. Zheng, J. Wen

Czułość i specyficzność analizy krzywych topnienia o wysokiej rozdzielczości w poszukiwaniu nieznanych polimorfizmów jednonukleotydowych i genotypowaniu znanych mutacji

S t r e s z c z e n i e

Analiza krzywych topnienia o wysokiej rozdzielczości (HRMA) została użyta do poszukiwania kandydujących polimorfizmów jednonukleotydowych (SNP) w eksonach genu *CAPN1* (duża podjednostka μ-kalpainy) kury. W analizie wykorzystano łącznie 312 próbek DNA od kur Beijing-you. Zaprojektowano dwanaście par starterów do amplifikacji dwunastu różnych eksonów, a polimorfizmy SNP stwierdzono w pięciu z nich. HRMA została również porównana z analizą PCR-SSCP w genotypowaniu znanych odcinków SNP w genie białka wiążącego kwasy tłuszczowe (*A-FABP*) adipocytów. Amplifikowane fragmenty DNA o długości 275 bp flankujące miejsce polimorficzne zostały pogrupowane przez PCR-SSCP w trzy genotypy: CC, TT i CT. Krótkie odcinki amplifikowanego DNA (56 bp), leżące w obrębie fragmentów o długości 275 bp, zostały użyte w celu zmaksymalizowania różnicy *T*m między homozygotami oraz w celu umożliwienia identyfikacji wszystkich trzech genotypów w pojedynczej analizie krzywej topnienia. Wyniki uzyskane różnymi metodami były weryfikowane krzyżowo a sekwencjonowanie losowo wybranych hetero- i homozygot wykazało specyficzność techniki HRMA. Pełna zgodność wyników wykazała, że HRMA jest użytecznym narzędziem szybkiego genotypowania miejsc polimorficznych w jednej próbówce (*closed tube*). Metoda ta umożliwia wydajne wykrywanie i skanowanie SNP na dużą skalę.