Three-step PCR and RFLP Genotyping of the Swine Ryanodine Receptor Gene Using Aged Single Hair Follicles Delivered by General Mail

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ABSTRACT : We have developed a reliable and noninvasive method for swine genotyping of single locus nuclear gene with aged single hair follicles delivered by general mail. The method is based on booster and nested PCR amplification with step-wise increase of primers and dNTPs concentrations followed by restriction endonuclease digestion. To establish this method, the ryanodine receptor (RYR 1) locus which is an economically important trait in swine industry was employed for genotyping experiment. The 3-step PCR amplication method is much less dependent on the quantity and quality of template DNA and produces enough amplification product for the detection on the ethidium bromide-stained gel such as RFLP analysis. A total of 120 pigs were subjected to the RYR 1 genotyping analysis using three-step PCR method which amplified enough quantity of PCR products from the aged single hair follicles for RFLP analysis and genotyping results were identical to the results of the corresponding ethanol-fixed skeletal muscle tissue. This approach will be a great help for porcine breeders and investigators in genotyping of swine. They can receive genotyping results later by simply plucking single hairs of their pigs at farm and sending them in general mail to the diagnostic laboratory which eliminates the inconveniences to collect ear tissue or blood cells from pigs, or the investigator's need for travel to farms in order to collect fresh hair samples. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 9 : 1237-1243)***

Key Words : Three-step (Booster and Nested) PCR Amplification, RFLP Analysis, Aged Hair Follicles, RYR 1 Locus, Genotyping

INTRODUCTION

The polymerase chain reaction (PCR) is one of the most widely used techniques of molecular biology. This technique allows enzymatic amplification of a specific region of DNA several million-fold and therefore, has even enabled the amplification and genetic typing of quite limited quantities of DNA such as DNA from single sperm or single diploid cells (Li et al., 1988). Single hairs are also valuable source of DNA for genotyping (Higuchi et al., 1988) and many genetic studies have been attempted with a variety of single hairs (Han et al., 1992; Lee and Chang, 1992; Taberlet and Bouvet, 1992; Uchihi et al., 1992; Ohhara et al., 1994; Needer and Liechti-Gallati, 1995; Wilson et al., 1995; Allen et al., 1998). However, DNA extracted from samples which have limited amount of template or partly degraded DNA demonstrated that an incorrect conclusion or failure of genotyping may be reached (Lee et al., 1991; Han et al., 1992). In order to improve the sensitivity and specificity of the amplification of target sequence when the available amount of template DNA is quite limited and template DNA contains very few copies of the target sequence, many refinements of the basic procedure have been developed and applied to a range of disciplines (Ruano et al., 1990; Richards et al., 1992; Taberlet et al., 1996; Neeser and Liechti-Gallati, 1995). Here, we describe a PCR amplification system for genotyping with aged single hair follicles received by general mail. The method using aged single hair follicles is particularly sensitive and thus well suited to analyze the genotype of single locus on the ethidium bromide-stained gel.

Ryanodine receptor (RYR1) is the calcium release channel of the sarcoplasmic reticulum in skeletal muscle whose contraction, relaxation and energy metabolism are regulated by the concentration of intracellular Ca^{2+} (MacLennan and Phillips, 1992). An abnormality in the ryanodine receptor facilitates opening and inhibits closing of Ca^{2-} channel resulting from an altered low-affinity Ca^{2-} binding site in the channel pore and thus causes malignant hyperthermia (MH) (Fill et al., 1990; MacLennan et al., 1990). MH-susceptible pig may be triggered to death by stress and if the animal experience acute stress prior to slaughter, large segments of the carcasses result in pale, soft, exudative (PSE) pork. These conditions are thus referred to as the porcine stress syndrome (PSS) which is economically serious problem in swine industry. cDNA encoding skeletal muscle ryanodine receptor was initially isolated from rabbit skeletal muscle sarcoplasmic reticulum (Takeshima et al., 1989) and cDNA encoding porcine skeletal RYR1 has been cloned (Fujii et al., 1991). Comparison of cDNA sequences encoding the Ca^{2-} release channels from normal and MH hogs indicated that nucleotide sequence at position 1843 in

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MH pig was substituted thymidine for cytosine leading to the substitution of Cysteine615 for Arginine615 in amino acid sequence (Fujii et al., 1991; Harbitz et al., 1992; Houde et al., 1993; Vogeli et al., 1994). The single nucleotide change at position 1843 of cDNA in MH pig eliminates a *Cfo* I restriction endonuclease recognition site in normal pigs, thus providing a straightforward procedure for detection of the mutation in genomic DNA amplified by PCR. This result allows to differentiate normal, heterozygous, and MH-susceptible swine in all breeds which provides breeders with the opportunity to eliminate MH gene from their herds and thereby removing the major cause of stress-induced death and PSE pork.

In general the template DNA of livestock in PCR genotyping is isolated from blood cells or ear tissue biopsies to obtain substantial quantities (Fujii et al., 1991; Hughes et al., 1992; Vogeli et al., 1994; Nakajima et al., 1996). The collection of ear tissues or blood cells results in the considerable stress to the animal and is very inconvenient. To alleviate these inconveniences, freshly plucked hair follicles were employed as an alternate source of template DNA. This attempt will allow genetic test of livestock much easier, cost effective and give less stress to animals. However, these sampling methods require researchers to take time away from laboratory to travel to pig farm. There is clearly a need for simpler and more cost-effective means of sample collection. We present three-step PCR coupled with restriction endonuclease-genotyping for the C-T mutation at nucleotide 1843 in the skeletal muscle ryanodine receptor (RYR1) gene using DNA extracted from swine aged single hair follicles delivered by general mail.

MATERIALS AND METHODS

Collection of samples

Single hairs were plucked from swine reared in farms and then either immersed in the fixation buffer (50% phosphate-buffered saline, pH 7.4, 50% ethanol) or sent to the laboratory by general mail without any treatment. The single hairs received by mail about 3-5 days after shipping were stored at room temperature for approximately 3 days. Muscle tissue samples of the corresponding pigs were collected at slaughter and stored in the fixation buffer. The ethanol-fixed samples were stored at 4°C in their fixation buffer until DNA extraction.

Extraction of DNA

Skeletal muscle tissue : Genomic DNA was isolated by incubation at 55°C overnight in 10 mM Tris-HCl, pH 8.0, containing 2 mM EDTA, 10 mg/ml DTT, 0.5 mg/ml proteinase K and 0.5% sodium dodecyl sulfate.

Single hairs : Single hairs stored in fixation buffer were washed twice with absolute ethanol and then once with distilled water. After drying in air, hair root portions (0.5 cm in length from the root end) were used for genomic DNA extraction. Genomic DNA was isolated and incubated at 55°C overnight in extraction buffer (10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 100 mM NaCl, 39 mM DTT, 200 μg/ml proteinase K and 2% SDS) (Uchihi et al., 1992). The aged single hairs delivered by mail were rinsed in distilled water. After drying, genomic DNA was isolated from hair root portion (0.5 cm in length from the root end) with extraction buffer and condition as above. These genomic DNA isolated from skeletal muscle, ethanol-fixed hair follicles and hair follicles delivered by mail were extracted with phenol and chloroform followed by precipitation with 2.5 volume of absolute ethanol and one-tenth volume of 3.5 M sodium acetate, pH 5.2. After washing with 70% ethanol, genomic DNA was purified by GENECLEAN II KIT, as recommended by manufacture (BIO 101 Inc, CA, USA).

Primers for PCR amplification

Two sets of primers were designed from the sequence of RYR1 gene. One set of primer, P-1 (forward primer: 5'-TCCAGTTTGCCACAGGTCCTACCA-3'), which corresponds to RYR1 genomic DNA nucleotide sequence at the position of 493 to 470 bases upstream from the mutation site (C/T) of RYR1 locus and P-2 (reverse primer: 5'-ATTCACCGGAGTGGAGTCTCTGAG-3'), which is complementary to RYR1 genomic DNA nucleotide at position of 141-164 bases downstream from the mutation site (C/T) of RYR1 locus, will amplify 659 base pair PCR product in length containing nucleotide at position 1843 (Vogeli et al., 1994; Nakajima et al., 1996). The other set of primers which are nested primers P-3 (forward primer: 5'-CATGTATGGACAACATCCACCT-3') corresponding to RYR1 genomic DNA nucleotide sequence at the position of 416-437 bases upstream from the mutation site (C/T) of RYR1 locus and P-4 (reverse primer: 5'-CTGGTGACATAGTTGATGAGGT-3') corresponding to RYR1 genomic DNA nucleotide sequence at the position of 64-85 bases downstream from the mutation site (C/T) of RYR1 locus will amplify 522 base pair PCR product in length.

PCR amplification conditions

Skeletal muscle tissue : PCR amplification was accomplished in 25 μl of reaction mixture, which contained 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 0.9 mM $MgCl_2$, 2.5 mM each of dNTPs, 50 pmole each of primers P-3 and P-4 (or 50 pmole each of primers P-1 and P-2), 100 ng genomic DNA, and 2.5 unit of Taq polymerase. The PCR reactions were carried out through 30 cycles of denaturation step at 94°C for 1min., annealing step at 53°C for 30 sec., and extension step at 72°C for 1 min.

Single hair follicles : The DNA amplifications were

performed in a three-step PCR. P-1 and P-2 primers amplifying 659 base pair in length were employed in the first and second steps and nested primers, P-3 and P-4 amplifying 522 base pair in length were introduced in the third step. The three-step PCR amplification reactions of 25 μl total volume in each reaction were performed as follows : The first-step PCR was carried out with the following components: 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.0 mM $MgCl_2$, 0.02 mM in each dNTP, 0.03 pmole each of primer P-1 and P-2, genomic DNA extracted from 5 single hair follicles and 2.5 unit Taq polymerase. In the second-step PCR, the concentrations of P-1, P-2 primers and dNTPs were 10-fold increased (i.e. 0.2 mM in each dNTP, 0.3 pmole each of primers P-1 and P-2) and 5 μl of the first-step PCR product was employed as a template. In the third-step PCR, 3 pmole each of primer P-3 and P-4 were introduced instead of P-1 and P-2 primers, concentrations of each dNTP were 2 mM and the template was 5 μl of the second-step PCR product. In the second-step and third-step PCR reactions, the concentrations of $MgCl_2$ and reaction buffer components were same as the one of the first-step PCR reaction. The PCR reactions in each of three steps were carried out through 25 cycles of denaturation step at 94°C for 1 min., annealing step at 53°C for 30 sec., and extension step at 72°C for 1 min.

Restriction digestion of PCR products with restriction enzyme, *Cfo* I

A 15 μl of PCR product was digested with overnight with 10 unit of *Cfo* I restriction enzyme in the buffer supplied by the manufacture at 37°C in a total volume of 20 μl. The digested PCR products were then run on a 2% agarose gels in TBE buffer (0.89 M Tris-HCl, 0.089 M borate, 2 mM EDTA, pH 8.0) containing ethidium bromide at 4 V/cm for 1 hour and the bands were visualized by UV light.

RESULTS AND DICUSSION

With the increasing amount and complexity of genetic testing, there is a constant need to seek easier and less expensive methods of sampling and subsequent genetic analysis. PCR technology has greatly simplified genetic analysis due to its requirement for only small amounts of crudely prepared DNA, but this technology can be further expanded upon to make genetic analysis even more efficient. In the present study we tested the feasibility of using aged swine single hairs delivered by general mail as a DNA source for 3-step PCR-RFLP based genotyping of single locus nuclear gene. RYR1 gene was selected as a single copy nuclear gene because point mutation at RYR1 locus causing PSS is economically serious problem in swine industry.

The appropriate conditions allowing amplification of RYR1 gene were initially determined using genomic DNA from ethanol-fixed muscle tissue samples. Figure 1 shows the PCR amplification products of RYR1 gene in the ethanol-fixed muscle sample with the primer set of P-1 and P-2 or the nested primer set of P-3 and P-4.

With the optimization of the PCR amplification conditions for the ethanol-fixed skeletal muscle sample, the concentrations of each primer set and dNTPs were adjusted to get decent amount of final PCR products from genomic DNA extracted from the ethanol-fixed or the aged single hairs.

Figure 2 shows the PCR amplification products of RYR1 gene from the ethanol-fixed muscle, the single hair follicles which were stored in the fixation buffer at 4°C for more than a year and the aged single hair follicles delivered by general mail, respectively.

The specific region of RYR1 gene was abundantly amplified in PCR reactions using DNA prepared from ethanol-fixed muscle tissue, ethanol-fixed single hair follicles and the aged single hair follicles which were delivered by general mail. There was no significant difference in the yield of amplification products among the different samples. It suggests that the single hair samples for PCR genotyping can be stored in the fixation buffer for a quite long period of time. In fact, DNA prepared from the aged hair follicles delivered by general mail yielded indistinguishable amounts of amplification product from DNA extracted from the ethanol-fixed single hair follicles.

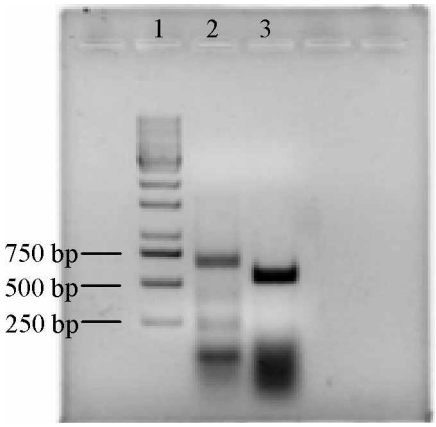

Figure 1. Single PCR amplification of RYR 1 locus with primer set of P-1 and P-2 or of P-3 and P-4 in the ethanol-fixed skeletal muscle sample. Lane 1 is molecular size marker with fragment sizes of 250, 500, 750, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 5,000, 6,000, 8,000, 10,000 bp. Lane 2 presents PCR product of RYR 1 locus with primer set of P-1 and P-2 and its product size is 659 bp. Lane 3 is PCR product of RYR 1 locus with nested primer set of P-3 and P-4 and its product length is 522 bp.

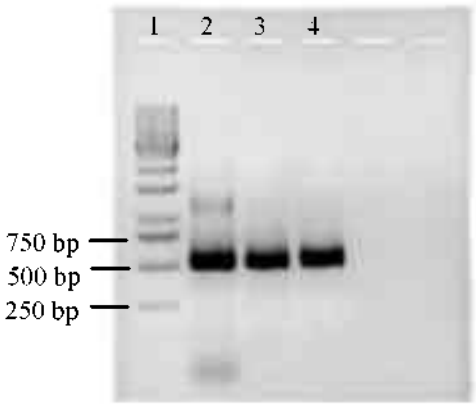

Figure 2. PCR amplification of RYR 1 locus in the ethanol-fixed skeletal muscle, the ethanol-fixed single hair follicles and the aged single hair follicles samples, respectively. Lane 1 is molecular size marker with fragment sizes of 250, 500, 750, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 5,000, 6,000, 8,000, 10,000 bp. Lane 2 presents PCR product of RYR 1 locus in the ethanol-fixed skeletal muscle sample with the nested primer set of P-3 and P-4 by single PCR amplification. Lanes 3 and 4 are amplification products in the ethanol-fixed single hair follicles and the aged single hair follicles, respectively. These PCR products were amplified by 3-step PCR including booster PCR using P-1 and P-2 primers and nested PCR employing P-3 and P-4 primers.

The amount of available template DNA from the aged single hair follicles delivered by general mail is very low, in the picogram range, though it is not accurately known. There was, however, no significant difference in the yield of amplification products among the different samples suggesting that 3-step PCR amplification method described in this paper is very useful for analysis on an ethidium bromide-stained gel when the available amount of template DNA is quite limited. It would be expected that amplification of single-copy sequences would require more template DNA than multiple-copy sequences to yield sufficient PCR product for detection on an ethidium bromide-stained gel. Sex determination by amplifying alphoid repeated sequences succeeded in a variety of forensic samples such as blood stains, viginal swabs, cigarett butts, bones and hair roots (Witt and Erickson, 1989; Neeser and Liechti-Gallati, 1995). The success of the genotyping with one-step PCR is based on the fact that the PCR amplification of repetitive sequences is much more sensitive than that of single-copy sequence. ABO blood type genotyping was successful with blood stains, saliva stains, semen stains, hair, bone tissue and semen contaminated with vaginal secretion (Lee and Chang, 1992). The PCR products whose sizes are 199-200 bp or 128 bp were digested with proper restriction enzymes and typed on an agarose gel. The success of genotyping with one-step PCR may partly due to the size of target DNA. There is a size requirement for efficient amplification from materials with limited DNA copy number (Allen et al., 1998; Ylitalo et al., 1995), and/or degraded DNA (Hagelberg et al., 1989; Paabo et al., 1988). For example, 450 bp is not efficiently amplified when DNA copy number is limited (Ylitalo et al., 1995). Amplification of over 200 bp was unsuccessful with DNA extracted from a 7000-year old human brain (Paabo et al., 1988). These results may explain why the one-step PCR product of 650 bp RYR 1 locus could not visualized on an agarose gel. The DNA content of aged single hairs is mostly degraded and quite limited. The amount of purified DNA has rarely been more than 200 ng from freshly plucked hairs and has usually been less than 10 ng from shed hairs, that is ~1,000 copies of the single copy nuclear gene (Higuchi et al., 1988). DNA typing from single hairs is, in most cases, classified into two categories. One is the one-step PCR amplification followed by dot blot hybridization with allele-specific oligonucleotide probes. For example, PCR products of single copy nuclear gene coding for human leukocyte antigen amplified from the root portions of single, shed hairs (Higuchi et al., 1988) or freshly plucked hair (Uchihi et al., 1992) were fixed on nylon membranes. Sequence specific oligonucleotide probes were used to type. The other category is PCR amplification followed by sequencing analysis. For instance, DNA extracted from the roots of bear single hairs collected in the field (Taberlet and Bouvet, 1992) or the human public hair shaft (Wilson et al., 1995). These DNA were subjected to amplify a control region of mitochondrial (mt) genome and an asymmetric PCR were performed to generate single-stranded DNA suitable for sequencing. The chief advantage of mtDNA typing over nuclear DNA typing is the high copy number of mtDNA per cell. However, these genotyping did not require restriction enzyme treatment and therefore require less amount of both the template DNA and the amplified DNA product compared to genotyping requiring restriction enzyme treatment such as PCR/RFLP analysis.

In swine, MH susceptibility results from the abnormality in calcium release channel from skeletal muscle sarcoplasmic reticulum (Nelson, 1983). The substitution of T for C 1843 in the RYR1 cDNA nucleotide sequence of MH susceptible pig leads to the change of Arg615 to Cys615 in the amino acid sequence. The nucleotide substitution leads to loss of a *Cfo* 1 restriction endonuclease site in the mutated RYR1 gene, thus providing a straightforward procedure for detection of the mutation in genomic DNA amplified by the PCR coupled with RFLP analysis. For analysis of the MH genotype using aged single hair follicles delivered by mail, genomic DNA isolated from the aged single hair follicles was amplified with booster PCR with primers of P1 and P2 producing 659 bp in length and then with nested PCR with primers of P3 and P4 resulting in the amplification product of 522 bp in length containing the the position of nucleotide 1843 of

cDNA encoding RYR1.

The quantity of genomic DNA extracted from the aged single hair follicles, though not precisely known, is quite low and varies greatly among samples. In order to improve the sensitivity of the PCR reaction when the template DNA contains very few copies of the target sequence, a two-step PCR, booster PCR or nested PCR, can be performed. The booster PCR amplification system was employed in detection of *Salmonella enteritidis* in feces from poultry. The first and the second PCR utilized the 0.2 μM and 1.0 μM of each primer, respectively. The booster PCR products were electrophoresed on an agarose gel for detection of the bacteria. The detection limit of the booster PCR was 1 cfu. per gram of feces (Cohen et al., 1994). In haplotyping of multiple polymorphism, the first-step PCR used very diluted genomic template and primer, and was subjected in 20 cycles of amplification. In booster PCR, primer was added to the first-step PCR product to make final concentration of each primer 100-fold compared to the first-step PCR reaction and 50 cycles of amplification was performed. For RFLP analysis, the PCR products were digested with restriction enzyme and then electrophoresed on an agarose gel. The gel, if necessary, was blotted on nylon membrane and hybridized to radiolabeled random hexamers. In addition to this, the booster PCR product was dotted on nylon membrane and hybridized to allele-specific oligonucleotide probes. It suggested that if the first-step PCR reaction contains quite low concentration of template and primer, artifactual formation of primer-dimmers can be reduced (Ruano et al., 1990). Sensitivity of the PCR assay for the detection of *Salmonella* or *Campylobacter* species showed that the detection limits for one-step PCR and nested PCR are 1,000 and 10 bacteria, respectively and therefore the sensitivity of nested PCR enhanced 100 times compared to one-step PCR (Wegmuller et al., 1993; Haedicke et al., 1996). Also, PCR detection results of *Listeria monocytogenes* in artificially contaminated raw milk indicated that one-step PCR could detect an average of 10,000 to 100,000 cfu. of bacteria in 1 and 25 ml of raw milk. Nested PCR enhanced the sensitivity of detection limit up to between 10 and 5 cfu. suggesting that sensitivity of nested PCR is about 1,000 times higher than that of a one-step PCR (Herman et al., 1995). In genotyping of a microsatellite locus using DNA extracted from a bear feces sample (Taberlet et al., 1996), the first-step PCR used diluted external microsatellite primers (0.01 μM) to reduce the formation of primer-dimer artifacts and the nested PCR introduced higher concentration of a nested primer (1 μM). The nested PCR analysis was performed for evidence materials of robberies consisting of shed hairs, saliva stains, saliva on stamps and sealed letters, nail scrapes and small bloodstains on cloth (Allen et al., 1998). PCR amplification systems are for mtDNA control region (D-loop)/second exon of nuclear HLA-DRB 1 gene. For typing, nested PCR products of mtDNA control region and the HLA-DRB 1 nuclear gene were sequenced and dot blot hybridization with allele-specific oligonucleotide probes. It also showed that nested PCR is 100 times more sensitive than one-step PCR. Thses results indicated that nested PCR can be 100 to 1,000 times more sensitive than one-step PCR.

In present study, the stress-susceptible allele is denoted "n" and the normal stress-resistant allele "N". For PCR-RFLP genotyping of RYR1 locus, nested PCR products were digested with *Cfo* 1 which recognizes the nucleotide sequence of the normal RYR1 genotype, N/N. If the nested PCR product is amplified from normal homozygote whose genotype is N/N, it will have been completely digested with *Cfo* 1 restriction enzyme and found 439 and 83 bp restriction fragments. If the product from normal heterozygote whose genotype is N/n, it will have been half digested and therefore produced 522, 439 and 83 bp fragments. If the PCR product is from MH-susceptible or PSS homozygote whose genotype is n/n, it will have no *Cfo* 1 cleavable site and therefore produce intact 522 bp fragment.

As seen in Figure 3, by examining the digested patterns of these amplified products, genotypes of RYR1 locus were easily determined. To evaluate the validation of the 3-step PCR with RFLP analysis for RYR1 genotyping, a total of 120 pigs were subjected to the 3-step PCR/RFLP test. All of PCR reaction yielded enough PCR products for RFLP analysis.

Figure 4 shows that both genotyping results from the aged single hair follicles and from corresponding muscle

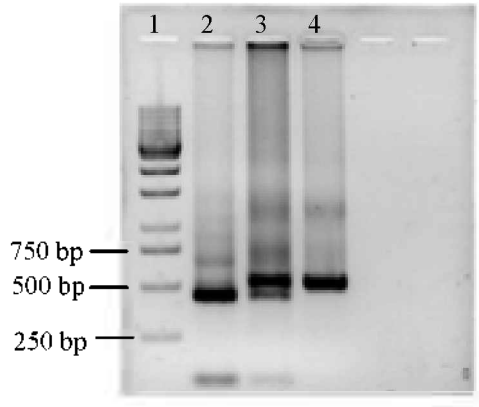

Figure 3. Restriction fragment patterns of three different RYR 1 genotypes (N/N, N/n, n/n) obtained with *Cfo* 1 restriction endonuclease digestion of the 3-step PCR amplification products on genomic DNA extracted from aged single hair follicles delivered by general mail. DNA samples were run on 2% agarose gel electrophoresis. Lane 1 is molecular size marker with fragment sizes of 250, 500, 750, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 5,000, 6,000, 8,000, 10,000 bp. Lane 2, 3 and 4 are samples with genotypes N/N, N/n and n/n, respectively.

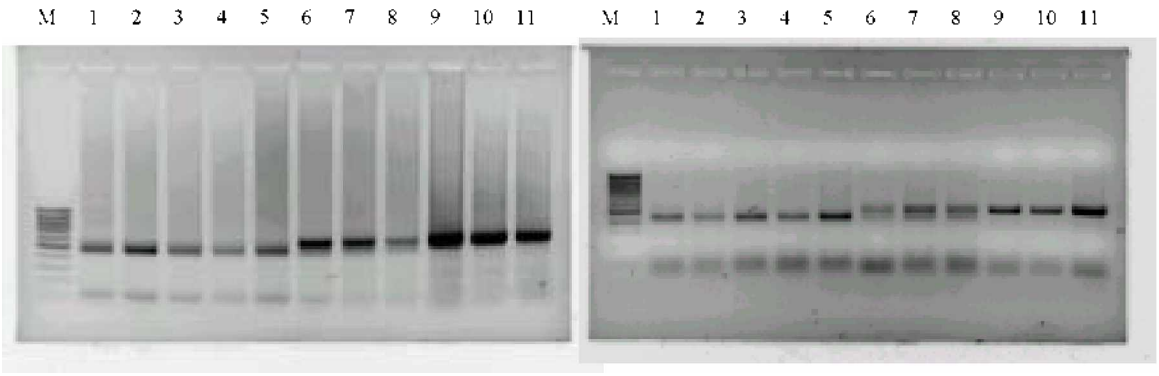

Figure 4. RYR1 genotyping results from the aged single hair follicles and from corresponding muscle tissue samples. A. RYR1 genotyping of pig with the aged single hair follicles. B. RYR1 genotyping of corresponding swine with muscle tissue samples. Arabic numerals on each lane indicate the individual pigs. Lane M is molecular size marker with fragment sizes of 250, 500, 750, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 5,000, 6,000, 8,000 and 10,000 bp.

tissue samples were identical to each other. It suggested that there was no preferential amplification of either allele of RYR1 locus from the quite limited amount of partially degraded genomic DNA extracted from aged single hair follicles. Table 1 shows the RYR1 genotypes of the aged single hair follicles from 120 pigs with 3-step PCR amplification and *Cfo* I restriction endonuclease analysis.

In this experiment, the success rate of PCR amplification with RFLP analysis is 100% for RYR1 genotyping. This result suggested that the 3 step PCR amplification method is much less independent on the quantity and quality of template DNA and therefore demonstrates that three-step PCR coupled with RFLP analysis is extremely reliable and suitable for single locus genotyping with aged single hair follicles delivered by general mail. An alternative method to three-step PCR coupled with restriction enzyme digestion is allele-specific PCR to detect a single base-pair substitution. In allele-specific PCR two allele-specific oligonucleotide primers, one specific for mutated allele and one specific for normal allele, together with another primer complementary to both alleles were used in the PCR with genomic DNA templates. For example, sickle cell anemia is caused by a point mutation which substitute T for A in the sequence encoding codon 6 of the human β-globin gene and this point mutation was diagnosed by allele-specific PCR (Wu et al., 1989). In this study we have addressed some of the technical issue of cost effective, sensitive and reliable genotyping analysis of single locus in animal. The results presented here showed that it may possible for swine rearing farmer or breeder to have genetic testing of their swine done by simply plucking single hairs of their pigs at farm and sending them by the general mail to the diagnostic laboratory and then receiving the genotyping results, eliminating the technician's need for travel to farms in order to collect samples for genotyping later. There are several advantages by using the approach presented in this paper for genotyping RYR1 locus. First, PSS genotype can be determined with single hair follicles which sent to the diagnostic laboratory. Second, no possible contamination of microorganism will cause ambiguous result. This approach will be a great help for porcine breeders and investigators in genotyping of swine. Another major advantage of this approach is that the simple, fast, economic, sensitive and reliable method makes it possible to be adopted in genotyping in human and animal.

Table 1. RYR1 genotyping analysis of the aged single hair follicles plucked from 120 pigs

Genotypes	No. of pigs
N/N	95
N/n	20
n/n	5
No amplification	0

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