

Effective Strategies for Identifying Novel Genetic Markers Based on DNA Polymorphisms

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Abstract

DNA markers offer a useful tool in human clinical medicine to predict disease susceptibility and drug response. In animal science, they can be applied in marker-assisted selection, gender determination, species identification or disease diagnosis. By combining DNA microarray technology and SNP (Single Nucleotide Polymorphism) genotyping, many novel genetic markers in candidate genes have been identified. These markers offer more advantages in that they can be used for accurate animal selection and to rapidly improve the reproductive performance over traditional breeding methods. Modified Random Amplified Polymorphic DNA (RAPD) or Random Amplified Microsatellite Polymorphism (RAMPO) fingerprinting were used for the study of DNA polymorphisms, and the results demonstrated that novel and stable markers can be used for animal gender determination and species identification. This paper presents effective strategies for identifying novel and stable markers based on DNA polymorphisms.

Keywords: Novel markers; DNA polymorphisms; Marker-assisted selection; Gender determination; Species identification

Introduction

DNA polymorphism analysis is often used for individual and paternity testing, gender determination, species identification, phylogenetic analysis, disease detection, marker-assisted selection and other genetic variation approaches. DNA microarray chip technology is a useful tool for investigating differentially-expressed genes in animals. Combining DNA microarray technology and polymorphism study, studies in our laboratory identified novel genetic markers of animals [1-8]. These candidate gene markers offer more advantages in that they can be used for accurate animal selection and to rapidly improve performance over traditional breeding methods. Two examples of genotypes of novel genetic markers associated with hatchability (%) in Tsaiya ducks (*Anas platyrhynchos*) are shown in Table 1. A number of methods for genetic polymorphism analysis were employed, such as Single-Nucleotide Polymorphism (SNP) [2-8], Random Amplified Polymorphic DNA (RAPD) [9-21], Random Amplified Microsatellite Polymorphism (RAMPO) [22,23], Amplified Fragment Length Polymorphism (AFLP) [24-27], Single Strand Conformation Polymorphism (SSCP) [28], Restriction Fragment Length Polymorphism (RFLP) [29,30] and mitochondrial DNA (mtDNA) sequence analyses [31,32]. In the present paper, effective strategies and applications for identifying novel and stable genetic markers based on SNP, RAPD and RAMPO polymorphisms were investigated.

Single Nucleotide Polymorphisms (SNPs)

SNPs are DNA sequence variations that occur when a single base in a gene is changed. SNPs in the coding regions of genes or in regulatory regions are more likely to cause functional differences than SNPs elsewhere [33]. SNPs usually occur in non-coding regions more

frequently than in coding regions. SNPs in non-coding regions may also have an impact on gene splicing, transcription factor binding or non-coding RNA. SNPs in the coding region are of two types, synonymous and nonsynonymous SNPs. Synonymous SNPs do not affect the protein sequence. A nonsynonymous variant causes an amino acid change in the corresponding protein. If a nonsynonymous variant alters protein function, the change can have drastic phenotypic consequences [34,35].

DNA microarray technology can be applied to carcinogen identification, toxicology, and drug safety [36]. This technology is also a useful tool for investigating differentially-expressed genes in animals [37-39]. Combining DNA microarray technology and genotyping, studies in our laboratory identified many novel genetic markers for animal selection [1-8]. Microscopic DNA spots are printed using pins or needles controlled by a robotic arm and attached to a solid surface. The DNA spots contain a specific DNA sequence, known as probes. These can be oligonucleotides, cDNA or small fragments of PCR products that correspond to mRNAs. Probe-target hybridization is usually detected and quantified by detection of fluorophore- or chemiluminescence-labeled targets to determine relative levels of transcripts. A flow chart of fabrication and analysis of DNA microarray for identifying novel SNP markers is shown in Figure 1. In order to identify differentially-expressed genes that are correlated with hatchability in the Tsaiya duck, a cDNA microarray-aided assay was performed. Total RNA samples from the magnum epithelium of 6 laying brown Tsaiya ducks with wide range variations in hatchability (44.00–81.82%) were collected and pooled for cDNA library construction [1]. Polymerase chain reaction was performed to amplify the cDNA inserts. The PCR products were

Candidate gene	Genotype performance			Reference
	+/+	+/-	-/-	
Ovomucoid	78.19 ± 1.99 ^a	74.60 ± 2.50 ^a	57.70 ± 5.38 ^b	Huang et al. 2011 [1]
Ovalbumin	79.59 ± 3.40 ^a	65.77 ± 2.07 ^b	76.35 ± 1.77 ^a	Huang et al. 2013 [3]

Table 1: Genotypes of novel genetic markers associated with hatchability (%) in Tsaiya ducks (Mean ± SE).

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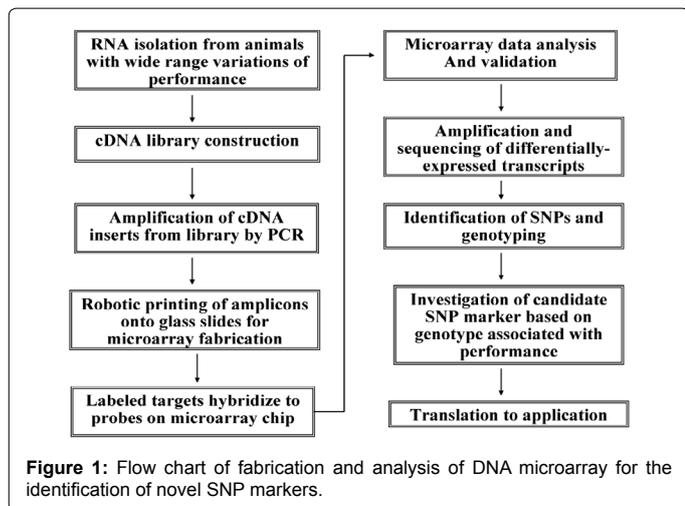


Figure 1: Flow chart of fabrication and analysis of DNA microarray for the identification of novel SNP markers.

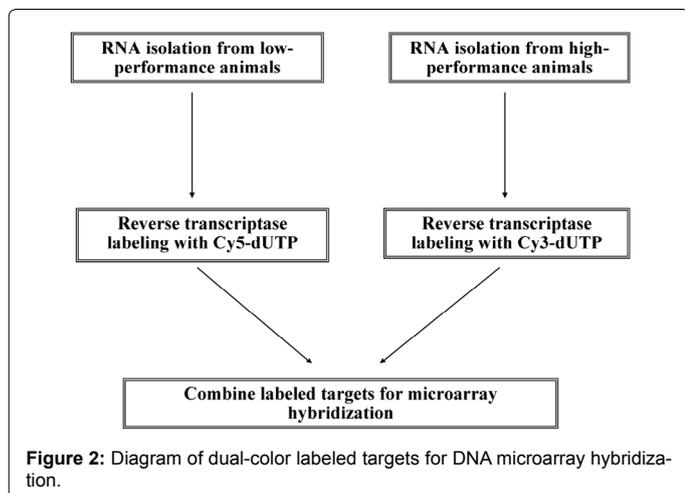


Figure 2: Diagram of dual-color labeled targets for DNA microarray hybridization.

subsequently purified by isopropanol precipitation, washed with 70% ethanol, resuspended in 20 μ l of 50% Dimethyl Sulfoxide (DMSO), and transferred to 384-well microplates as microarray sources for printing [38,39]. Printing was performed under the conditions of 60% relative humidity and a 300- μ m dot space [37]. The 2912 amplicons were printed onto GAPS II-coated glass slides (Corning Life Sciences, Corning, NY, USA) using an OmniGrid Accent™ microarrayer (GeneMachine, Bethesda, MD, USA); each amplicon was spotted in triplicate, resulting in 8736 spots on each slide [1]. The fluorophore-labeled targets hybridize to probes on the microarray and hybridization signal scanning was carried out. Differentially-expressed transcripts associated with hatchability were found.

Dual-color labeled targets are widely used for microarray hybridization. The procedure of dual-color labeling is shown in Figure 2. The total RNA samples were collected from the magnum epithelium of high-(88.20 \pm 1.21%, $n=5$) and low-hatchability (55.60 \pm 1.04%, $n=5$) ducks, and reverse transcriptase labeling was performed with Cy5- and Cy3-dUTP, respectively. The two Cy-labeled cDNA samples were mixed and hybridized to a single microarray that was then scanned in a microarray scanner to visualize the fluorescence of the two fluorophores after excitation with a laser beam of a defined wavelength. The relative intensities of each fluorophore may then be used in ratio-based analysis to identify up-regulated and down-regulated genes

[3,40]. Most two-color microarray experiments suffer from systematic differences in the red and green intensities that require correction [41]. Dye-swap experiments can reduce systematic bias due to the affinity difference of the fluorescent dyes and achieve more reliable cDNA microarray analysis results (Figure 3). The Cy5-dUTP- and Cy3-dUTP-labeled targets are commonly used for hybridization with probes on a microarray. When possible, using dye-swap pairs is recommended.

To confirm the results of microarray analysis, real-time polymerase chain reaction or Western blot analysis were used to validate the candidate gene expression [1-3]. The fragment of the differentially-expressed gene will be amplified by PCR from the genomic DNA templates and sequenced for SNP identification. Sequences were screened against the NCBI nucleotide database using the BLASTN program (<http://www.ncbi.nlm.gov/BLAST/>) and aligned using the GCG software alignment program (Genetics Computer Group, Madison, WI, USA). Alternatively, SSCP analysis is a simple and more sensitive method for the detection of mutations in DNA sequence to find SNP site based on PCR technology. It can be performed before nucleotide sequencing to save cost and time. [6-8]. Individuals showing different SSCP profiles

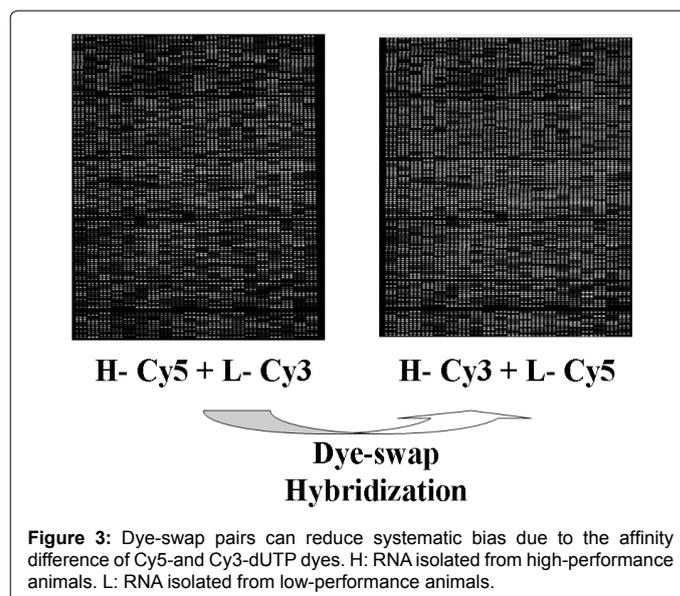


Figure 3: Dye-swap pairs can reduce systematic bias due to the affinity difference of Cy5- and Cy3-dUTP dyes. H: RNA isolated from high-performance animals. L: RNA isolated from low-performance animals.

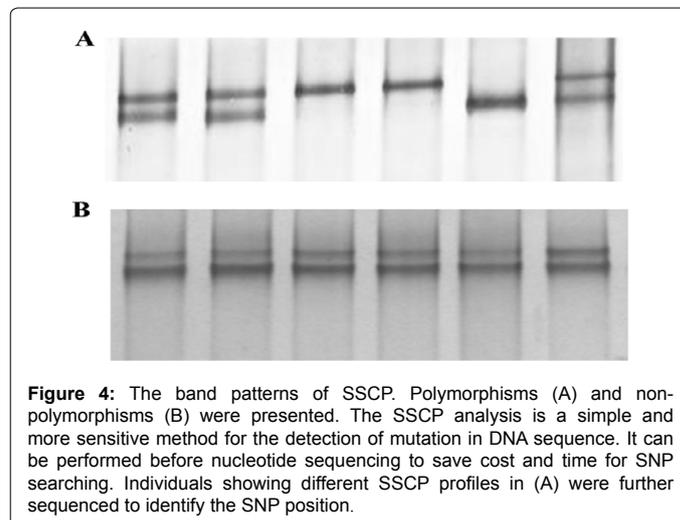
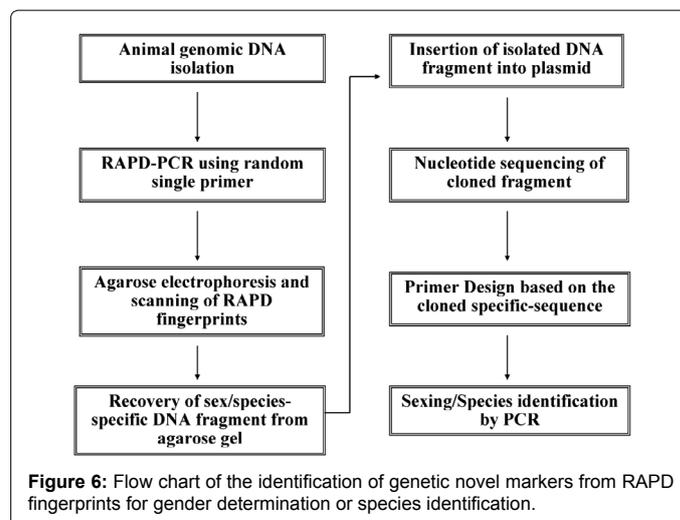
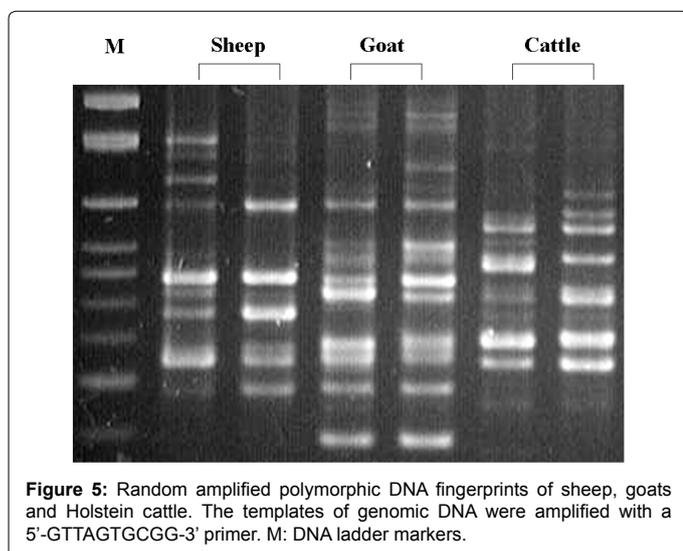


Figure 4: The band patterns of SSCP. Polymorphisms (A) and non-polymorphisms (B) were presented. The SSCP analysis is a simple and more sensitive method for the detection of mutation in DNA sequence. It can be performed before nucleotide sequencing to save cost and time for SNP searching. Individuals showing different SSCP profiles in (A) were further sequenced to identify the SNP position.

were further sequenced to identify the SNP position (Figure 4). SNP genotypes can be detected by PCR-RFLP or minisequencing. All data were subjected to ANOVA using the general linear model procedure in the SAS statistical package version 9.1.3 (SAS Institute Inc., Cary, NC, USA), followed by Duncan's multiple comparison testing for genotype-trait association analysis and confirmation by the Chi-square test [3].

Random Amplification of Polymorphic DNA (RAPD)

RAPD fingerprinting is a very fast method to investigate genetic variation. It is amplified by PCR from the template of genomic DNA with a single and short random primer (8-12 nucleotides). RAPD-PCR is different from the standard PCR to amplify the template DNA with two primers. It does not require any specific knowledge of the target genomic template sequence; multilocus polymorphic markers generated by RAPD-PCR allow the examination of genomic variation [42]. The random primer will bind somewhere in the complementary sequence of the template genomic DNA using RAPD-PCR amplification. The PCR products are detected by electrophoresis in agarose gel and stained with ethidium bromide. RAPD fingerprints are saved using an image analysis system [15]. RAPD-PCR is easy to perform and does not require expensive equipment or the use of a radioactive isotope. The bands of RAPD fingerprints often show high polymorphisms in animal species. Selecting the right sequence for the primer is very important because different sequences will produce different band patterns and possibly allow for more specific recognition of animals. In other words, the band patterns of RAPD fingerprints depend on the animal individuals, species and random primers. RAPD fingerprints are usually dominant, as polymorphisms are detected as the presence or absence of bands after polymerase chain reaction. In Figure 5, the genomic DNA of sheep, goats and cattle was amplified by PCR with a 5'-TTCCCCCAG-3' primer. The results showed that the band patterns of the RAPD fingerprints were different among species, but some major bands looked similar within species. Different genetic backgrounds of these three species generated different fingerprint patterns. The band polymorphisms derived from different templates of genomic DNA in different species and the primer-binding sites were also different, which led to the production of different lengths of RAPD-PCR products. The RAPD technique might offer the possibility of carrying out compatibility analysis with unlimited number of primers, each detecting variation at several regions of the genome [15].



As RAPD fingerprints are well-known to be sensitive to reaction conditions, such as the quality and concentration of the template DNA, primer, reagents, PCR cycling conditions, ramping speed and the type of PCR instrument, which may greatly influence the PCR products [22,43]. Thus, RAPD fingerprinting requires carefully-developed laboratory protocols in order to be reproducible. Longer, sequence-complementary primers require a more stringent annealing temperature than that used with the RAPD assay. Due to problems in experiment reproducibility, it is advisable to clone the RAPD marker and convert it into sequence-characterized amplified regions. The amplification of the cloned specific sequence can provide a method for genetic marker study [9-21,44].

In our laboratory, we isolated genomic DNA from animals; RAPD-PCR was performed to amplify the template DNA using a random primer, and the PCR products were run in agarose gel by electrophoresis to separate the amplified DNA fragments. The specific band was recovered from the polymorphic bands of the RAPD fingerprints in agarose gel. The DNA fragment of the recovered specific band was constructed into a plasmid vector. Nucleotide sequencing of the cloned fragment was carried out. Specific primers were designed based on the cloned specific-sequence. The primers were used for PCR to amplify the template genomic DNA for gender determination or species identification (Figure 6). This can be used for identifying animal novel genetic markers. We applied this procedure successfully to search for many novel genetic markers for gender determination in geese [16], pigs [10], cattle [11], pigeons [13], ostrich [18,19], Columbidae birds [20] and ducks (Figure 7) in our laboratory. We also used this technique successfully to identify the pig-specific genetic markers (Figure 8) and cattle, goats, ostrich, emu, chickens, turkeys, geese, pigeons, etc. [17] and Mugilidae [21] for species identification. The results all showed that the sex or species of these animals could be accurately and rapidly identified by normal standard PCR.

Random Amplified Microsatellite Polymorphisms (RAMPOs)

RAMPO fingerprinting was reported by Richardson et al. [22]. This method is a combination of RAPD-PCR and microsatellite-complementary oligonucleotide probe hybridization. The genomic DNA samples were isolated from animals; the single random primer was employed for RAPD-PCR to amplify the template genomic DNA, and the PCR products were separated by agarose gel electrophoresis and

stained with ethidium bromide. RAPD fingerprints were saved using an image analysis system, then the agarose gel DNA was transferred onto nylon membrane. The simple repeat sequence of $(TG)_6$ was used as

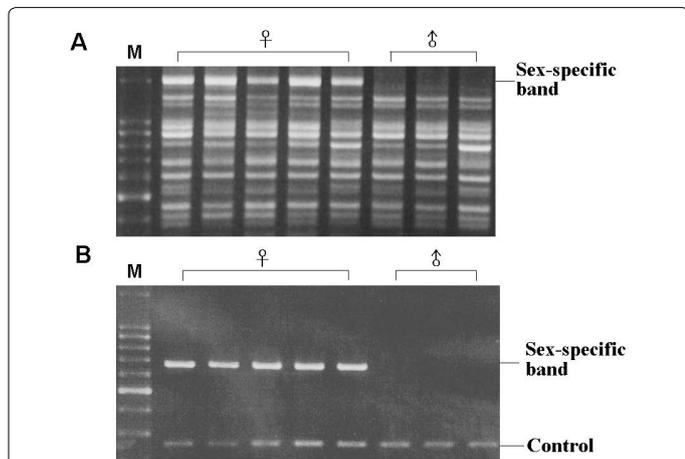


Figure 7: An example of gender determination by standard PCR amplification using sex-specific primer sequences cloned from RAPD gel. A. Random amplified polymorphic DNA fingerprints of female and male ducks. B. Duck genomic DNA was used as template and amplified by standard PCR with sex-specific primers. Sex-specific bands on agarose gel were present in females only. M: DNA ladder markers; Control: amplified 18S ribosomal gene fragments.

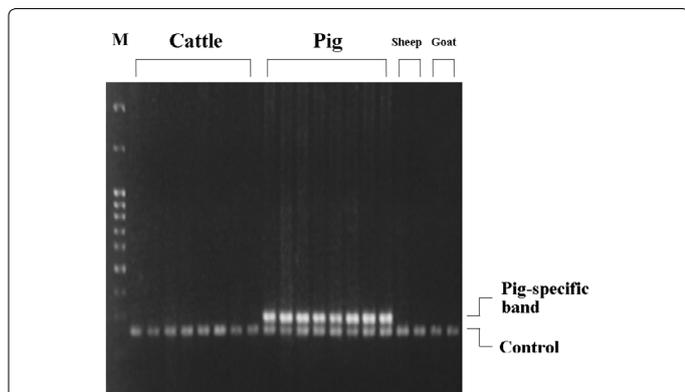


Figure 8: An example of species identification by standard PCR amplification using species-specific primer sequences cloned from RAPD gel. Genomic DNA of cattle, pigs, sheep and goats was used as templates and amplified with pig-specific primers by PCR. The species-specific bands on the agarose gel were present in pigs only. Control: amplified 18S ribosomal gene fragments. M: DNA ladder markers.

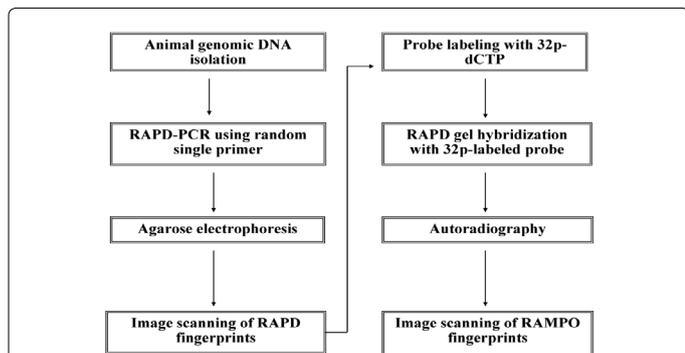


Figure 9: Flow chart of Random Amplified Microsatellite Polymorphism (RAMPO) fingerprinting.

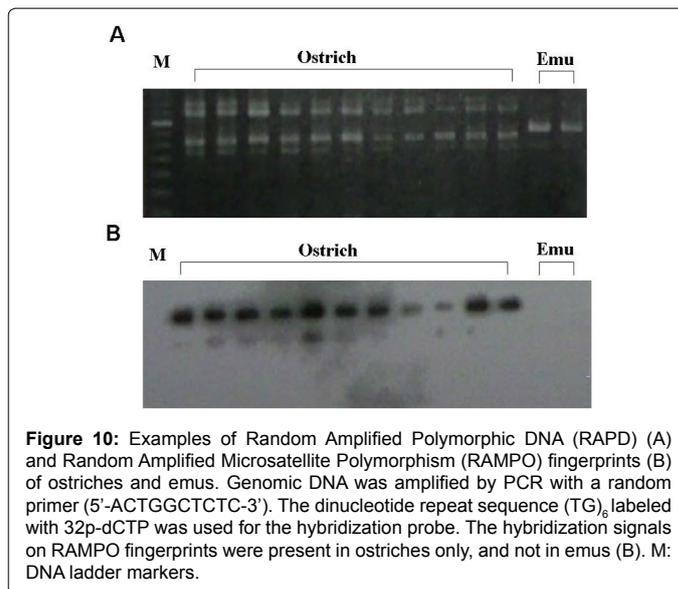


Figure 10: Examples of Random Amplified Polymorphic DNA (RAPD) (A) and Random Amplified Microsatellite Polymorphism (RAMPO) fingerprints (B) of ostriches and emus. Genomic DNA was amplified by PCR with a random primer (5'-ACTGGCTCTC-3'). The dinucleotide repeat sequence $(TG)_6$ labeled with ^{32}p -dCTP was used for the hybridization probe. The hybridization signals on RAMPO fingerprints were present in ostriches only, and not in emus (B). M: DNA ladder markers.

the probe, labeled with ^{32}p -dCTP and hybridized with DNA on nylon membrane. The nylon membrane was then placed into a cassette with an intensifying screen and covered with X-ray film for autoradiography. The RAMPO fingerprints were saved and analyzed after the X-ray film was developed (Figure 9). Alternatively, the agarose gel containing DNA can be dried at $80^{\circ}C$ in vacuum machine for gel hybridization to save the cost of nylon membrane. In the other words, the gel containing DNA can be either dried directly or Southern transferred onto nylon membrane for hybridization. For the purpose of moving away from the use of hazardous radioactive isotopes, the Digoxigenin (DIG) system can enable nonradioactive and highly sensitive detection of nucleic acids. Nonradioactive technology therefore offers a choice to label and detect nucleic acids for the application of hybridization.

When the RAPD and RAMPO fingerprints were compared in Holstein cattle, the results showed that the band patterns either in the RAPD or RAMPO fingerprints presented polymorphisms, but the patterns of the fingerprints in both were different. The number of bands in the RAPD fingerprints was greater than that in the RAMPO fingerprints. The patterns of the fingerprints in both were different, which depended on the primer sequences. A band appeared in the RAPD fingerprints, and sometimes disappeared in the RAMPO fingerprints. This indicated that the DNA sequence of the band of the same site in RAPD fingerprints probably different from that of the band in RAMPO fingerprints. The RAPD bands were slight or smeared, but strong signals were represented in the RAMPO fingerprints [9,45].

RAMPO fingerprinting can be used to identify new gender or species markers for animal gender determination or species identification. We found a novel male-specific marker by RAMPO fingerprinting in Holstein cattle [23]. This technique can also be used to identify novel species-specific genetic markers. Figure 10 shows an example of RAPD (A) and RAMPO (B) fingerprints of ostriches and emus. Genomic DNA was amplified with a random primer (5'-ACTGGCTCTC-3'). We used a dinucleotide repeat sequence $(TG)_6$, a microsatellite-complementary oligonucleotide sequence, for the hybridization probe labeled with ^{32}p -dCTP. In both the ostriches and emus, the band patterns of the RAPD fingerprints presented polymorphisms, but there were some variations in their genetic constitutions, which can be expected (Figure 10A). These profiles of RAMPO fingerprints were completely

different from the RAPD staining patterns, and strongly depended upon the hybridization probe. Distinctly different RAMPO fingerprints between ostriches and emus were produced when RAPD gel DNA was hybridized with the 32p-labeled (TG)₆ probe. The hybridization signals in the RAMPO fingerprints resulted from PCR products and not from unamplified genomic DNA that might be present in the gel. The signals generated by hybridization with the 32p-dCTP labeled (TG)₆ probe provided new polymorphisms that were different from those seen after ethidium bromide staining of RAPD bands. The RAPD products could hybridize with the 32p-dCTP labeled dinucleotide repeat sequence (TG)₆ in ostriches only, but no in emus (Figure 10B). The results revealed that this method can be used to distinguish the species of ostriches and emus according to the patterns of the RAMPO fingerprints.

Conclusion

DNA polymorphisms can be single base variations, deletions, or insertions in a specific genome. Different types of DNA polymorphisms allow us to identify a genetic marker if a specific DNA polymorphism is linked to a specific trait. Combining DNA microarray technology and SNP genotyping, or modified RAPD and RAMPO fingerprinting, this paper describes highly desirable tools for developing novel and stable genetic markers based on DNA polymorphisms. These effective strategies will be of great help in identifying more novel and stable markers in the future.

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