Development of Sequence Characterized Amplified Regions (SCAR) Showing for Cheju Native Horse

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This study was conducted to analyze genetic characteristics and to develop the specific marker for Cheju native horse (Cnh) at the level of sequence characterized amplified regions (SCARs). We collected blood samples from Cheju native horse and Thoroughbred horse (Th) and obtained genomic DNA from the blood of 50 individuals randomly selected within the breeds. Seven hundred primers were chosen randomly and were used to examin the polymorphism and 40 kinds of primers showed polymorphic RAPD band patterns between two breeds. Thirty primers of them showed horse specific bands. With the primer MG 30, amplified band of 2.0 kb showed the specificity to Cheju native horse (Cnh). Additionally MG 53 detected the thoroughbred horse (Th) specific markers at size of 2.3 kb. As the next, 2.3 kb band from MG 53 was checked with the all individuals from all the breeds of this study, and it maintained the reproducible breed specificity to thoroughbred horse (Th). With this results, 2.3 kb band was cloned into plasmid vector and sequenced bidirectionally from both ends of the cloned fragment. With the obtained sequences 10 nucleotide extended primers including the original arbitray primer were designed as a SCARs primer. Finally, the primer with extended sequence showed the reproducible breed differentiation pattern and it was possible to identify Cheju native horse (Cnh) from other breeds. The SCARs marker 2.3 kb from MG 53 could be used to identify Cheju native horse (Cnh) for not only registration but also horse breeding programe.

Key words – Cheju native horse (Cnh), DNA Marker, Thoroughbred (Th), RAPD-PCR, SCAR

With the advent of molecular biological techniques, DNA-based approaches for genetic analysis have been proposed for genetic marker of numerous organisms. The genetic maker system with DNA polymorphism was considered to be more correct methodology in measurement of genetic difference bewteen breeds and individuals than conventional system. Therefore, many and wide range of application has been accomplished with these techniques including establishment of genetic identity, population genetics, forensic applications, paternity test, identifying the loci related to economic traits, construction of genome map, conservation biology, identification of transformed cell line and so on[6,7,15]. For these purpose, lots of approaches to detect polymorphic DNA sites has been applied and new specified techniques was developed. RFLP (Restriction Fragment Length Polymorphism) and DNA fingerprinting (DFP) are techniques of great importance in identifying DNA polymorphisms. As an another valuable resource for genetic analysis and DNA profiling methods, microsatellite (MS) or short tandem repeats (STR) derived makers have developed.

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As an alternative approach for the detection of DNA polymorphisms of the genome[8,10,16,17] reported a novel DNA analysis technique based on the amplification of random DNA sequences using the polymerase chain reaction (PCR) and single primers of arbitrary sequences. DNA variation from randomly amplified polymorphic DNAs (RAPD, arbitrary primed-PCR) has recently been helpful in addressing a wide range of evolutionary problems including the deter-mination of paternity[12,14]. It is currently used in numerous organisms to construct detailed genetic maps, tag important genes, and for population genetic studies[2,3,4,8,9]. The advantages of RAPD over RELP or DFP are their simplicity and rapidity. They do neither require the use of radioisotopes nor the construction of genomic library and, most of all, only very small amounts of genomic DNA are needed. Other approaches that may useful in detecting polymorphism in SCAR include denaturing gradient gel electrophoresis[11] and PCR-single strand conformation polymorphism analysis[5]. Therefore, a codominant marker may be produced from an initially dominant RAPD marker. The creation of SCAR marker from a RAPD marker of interest also eliminates concern regrading reaction reproducibility and scoring of similar-sized, nonhomologous fragments. Therefore, the SCAR technique using short oligonucleotide

sequences was useful for genetic analysis in horse. In this study, we used SCAR marker to characterize genetic background and develop specific genetic marker for Cheju native horse (Cnh).

Materials and Methods

Genetic stocks and DNA preparation

Experimental animals were the two horse breeds; Cheju native horse (Cnh) and Thoroughbred horse (Th). Cheju native horse is phenotypically very different and thought to have unique genetic background from other foreign breeds. Fifty individuals randomly selected from each breed were analyzed. Genomic DNA was prepared from whole blood samples collected from 50 Cheju native horse (Cnh) and 50 Thoroughbred (Th). Genomic DNAs from the samples were extracted using a Wizard Genomic DNA Purification Kit (Promega, USA)

Random amplified polymorphic DNAs

Random amplified polymorphic DNAs (RAPD) analysis was performed as outlined by Williams et al[16]. The optimized condition was as follow; twenty five microliter of reaction mixture consisted of fifty nanograms of genomic DNA, 1×reaction buffer (50 mM KCl 10 mM Tris-Cl pH 8.3, 1.5 mM $MgCl_2$, 0.01% Triton X-100, supplied by manufacturer), dNTP (each 200 μM), 0.5 unit of Taq DNA polymerase (Takara, Japan) and 100 nM of primer (purchased from University of British Columbia, Canada, and Operon, USA) and then overlaid same volume of mineral oil. The G+C contents of these 10 oligonucleotide primers were 50~80%. Amplification was conducted in Perkin Elmer Cetus DNA Thermal Cycler. Initial denaturation was performed for 5 min at 95℃ and followed by 50 cycles of denaturation at 95℃ for 1 min, annealing at 39℃ for 1 min, extension was at 72℃ for 2 min. Final extension was carried out at 72℃ for 7 min after cycles were completed.

Breed-specific band cloning and sequencing

The selected RAPD fragment isolated from the gel and cloning of the amplified breed specific fragment were processed according to the procedures of the pGEM-T Easy Vector System (Promega, USA). Ligated plasmid was transformed to *E.coli* (JM109 or DH5α) by $CaCl_2$ method. Plasmid DNA was prepared by Quiagen plasmid kit (Quiagen, Germany) and sequenced by Perkin Elmer dye terminator sequencing kit (PE Applied Biosystems) using Perkin Elmer automatic sequencer (ABI 377; PE Applied Biosystems).

Design of SCAR primer

After determining of the cloned breed-specific fragment sequence, strand-specific oligonucleotide primers which added to the 3'-terminus of random primer (8 to 12 bp) were designed based on the sequence information (Bioneer Co. Korea).

Results and Discussion

In order to examin the extent of genetic variability using the RAPD marker in horse population, seven hundred primers of random sequences were obtained from commercial suppliers (University of British Columbia, USA) and screened for the appropriate amplification pattern. Fig. 1 shows the polymorphic RAPD patterns between breeds of horse with 3 different primers. It showed primer-specific pattern, and we could identify higher polymorphism in band pattern generated MG-562. Out of 700 primers tested, 410 amplified identifiable products and out of them only 100 primers generated different band patterns between breeds of horse. And finally 30 primers were selected for the genetic analysis in horse. Using 410 primers, we were able to detect average 9 bands per primer which ranged from 0.2 kb to 3 kb in size, and with the selected primers 270 polymorphic bands were obtained. Therefore, it seemed that the condition used in this study has lower stringency for primer annealing in comparison to other report of average 4.6 bands per primer plotsky et al[13].

Fig. 2 and 3 are the amplification profiles for two horse breeds with each a single different primer. Fig. 2 shows the higher polymorphic band pattern comparable with DNA fingerprints. Although, in some cases, high variability in band pattern could be derived from the inherent sensitivity in amplification condition of RAPD, MG6085 showed reproducible band pattern. Therefore, it was considered to be useful for genetic analysis. Fig. 3, 4, showed another polymorphic markers of RAPD products. RAPD band pattern of Th was clearly different from this of Cnh. These results mean that target

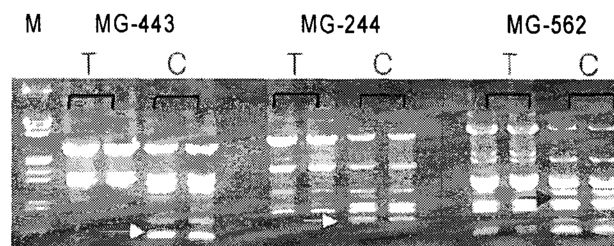


Fig. 1. DNA amplification profiles generated with random primers MG-443 MG 244, MG 562 on two breeds of horse. (C: Cheju native horse, T: Thoroughbred, M: Lamda / *Eco*RI+*Hind* III.)

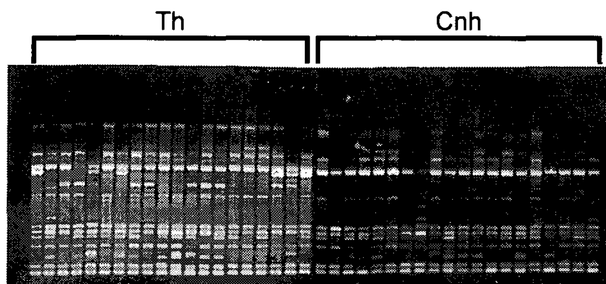


Fig. 2. DNA amplification profiles generated with random primers MG 6085, on two breeds of horse. (Cnh: Cheju native horse, Th: Thoroughbred, M: Lamda / *Eco*RI+*Hind* III.)

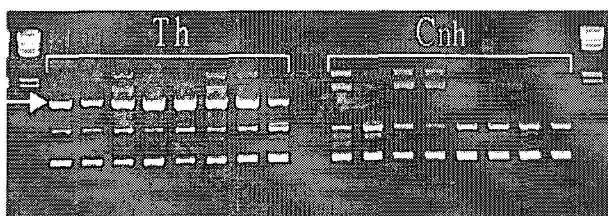


Fig. 3. Breed specific RAPD markers of primer MG44 in horse. (Cnh: Cheju native horse, Th: Thoroughbred, M: Lamda / *Eco*RI+*Hind* III.)

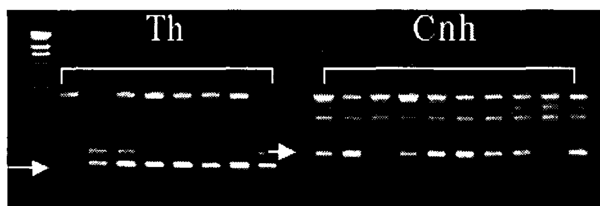


Fig. 4. Breed specific RAPD markers of primer MG162 in horse. (Cnh: Cheju native horse, Th: Thoroughbred, M: Lamda / *Eco*RI+*Hind* III.)

DNA sequence to which random primer annealed were not same in Th with Cnh. Therefore, these RAPD bands specific to breed have a potentials possibility for genetic marker in specific breed. In Fig. 5, breed specific RAPD bands which present only in Th, were identifiable as indicated by arrow. Band was approximately 2,300 bp in size and appeared in all 5 Thoroughbred horses (indicated as Th), but was not observed in Cnh. Fig. 6 shows polymorphic marker to Cnh horse. These breed specific RAPD bands will be also very useful putative genetic markers to identify Cnh from other breeds. For more obvious conclusion, however, more number of animals within each breed must be tested in RAPD polymorphism study.

Also, this study supported the previous reports by Bailey & Lear[1] that RAPD method with short oligonucleotide primers was useful for genetic analysis in horse. Thirty primers of them showed RAPD band pattern specific to breed. With the primer MG 30, amplified band of 2.0 kb showed the specificity to Cnh (Fig. 6). Additionally MG 53 detected Th specific markers at size of 2.3 kb (Fig. 5). As the next, 2.3 kb, 2.0 kb bands from MG 53 and MG 30 were checked with the all individuals from all the breeds of this study, and it maintained the reproducible breed specificity to Th and Cnh. With this results, 2.3 kb and 2.0kb bands were cloned into plasmid vector and sequenced bidirectionally from boths ends of cloned fragment.

With the obtained sequences 10 nucleotide extended primers including the original arbitray primer were designed as a SCARs- primer (MG53T23 and MG30J20). The primer with extended sequence showed the reproducible breed differentiation pattern (Fig. 7, 8), and it was possible to identify Cnh from other breeds, SCARs markers from MG 53T23 and MG 30J20 could be used to identify Cnh for registration as well as horse breeding programe.

Therefore, the SCAR-PCR technique using short oligonucleotide sequence was useful for genetic analysis in horse.

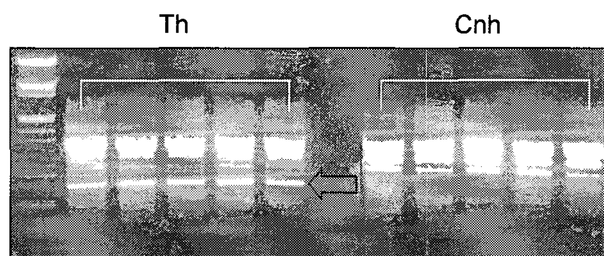


Fig. 5. Breed specific RAPD markers of primer MG 53 in horse. (Cnh: Cheju native horse, Th: Thoroughbred, M: Lamda / *Eco*RI+*Hind* III.)

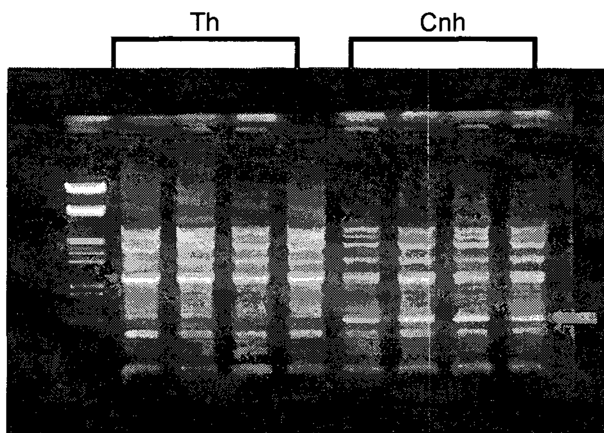


Fig. 6. Breed specific RAPD markers of primer MG 30 in Horse. (Cnh: Cheju native horse, Th: Thoroughbred, M: Lamda / *Eco*RI+*Hind* III.)

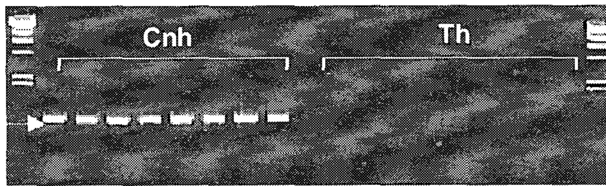
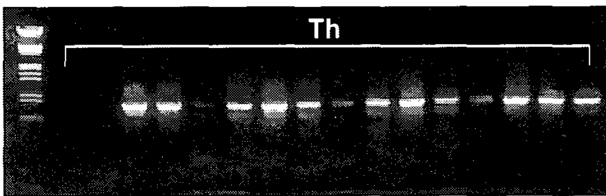


Fig. 7. Agarose gel separation (1.2%) of PCR amplified DNA with Cnh-SCAR primer drived from MG 30 RAPD fragment. {Cnh: Cheju native horse, Th; Thoroughbred M: Size marker (Lamda / *Eco*RI+*Hind* III digested.)}



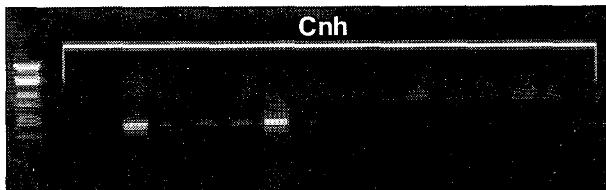


Fig. 8. Agarose gel separation (1.2%) of PCR amplified DNA with thoroughbred horse-SCAR primer derived from MG 53 RAPD fragment. {Cnh: Cheju native horse, Th: Thoroughbred M: Size marker (Lamda / *Eco*RI+*Hind* III digested.)}

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초록 : 품종 특이성을 이용한 제주마 판별 표지인자 개발

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본 연구는 RAPD 기법을 이용한 종 특이 marker 개발 및 이 marker의 SCAR marker로의 개발을 목표로 수행되었다. Random primer 700개에 대하여 PCR 수행결과, 품종간, 개체간에 많은 다형성이 관찰되었으며 품종특이적인 양상을 나타내는 MG30, MG53의 primer는 각각 2.0 kb, 2.3 kb의 위치에서 제주말과 더러브렛종의 특이적인 RAPD 단편을 나타내었다. 이들 단편들 중 품종 특이적인 단편을 클로닝한 후 random primer가 포함된 부분의 염기서열을 결정하였다. 10 bp의 RAPD random primer에 10 bp의 염기를 추가하여 SCAR primer를 제작하였다. SCAR marker의 수행결과 RAPD marker와 같은 2.3 kb, 2.0 kb의 크기에서 제주마와 더러브렛종에 특이적인 하나의 밴드가 증폭되었다. 따라서 이 Cnh -SCAR marker는 보다 안정적이고 재현성 있는 marker로서 사용이 가능하여 제주말의 판별에 유용하게 사용될 수 있을 것이다.