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EVALUATING THE USEFULNESS OF POLYMORPHISM OF 7 MICROSATELLITE DNA MARKERS FOR GENETIC DIVERSITY STUDIES OF SHEEP

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Abstract

The aim of the study was to determine the polymorphism of some microsatellite sequences in Wrzosówka sheep using the multiplex PCR reaction. Seven markers recommended by FAO for analysis of biodiversity were studied: BM757, BM6526, OarCP20, OarCP34, OarFCB128, OarFCB304, and OarHH35. One of each starter pairs was labelled with a fluorescent dye: FAM, TET and HEX. The main factors that influenced the obtainment of PCR products was the determination of annealing temperature (58°C) and primer concentration in the reaction mixture (from 0.3 to 0.6 μM). DNA fragment sizes were determined in an ABI 377 DNA sequencer. In the animals studied, PCR products were obtained from all loci analysed. The number of alleles detected ranged from 5 for locus BM757, BM6526 and OarCP34 to 8 for OarFCB128. The results suggest that the 7-plex PCR reaction and automated DNA sizing technology can be applied to investigate genetic variation in sheep.

Key words: sheep, microsatellite DNA, PCR multiplex

The genetic structure and characteristics of various breeds of sheep, especially those threatened with extinction, is attracting increasing international attention, taking into account the largest number of genetic markers possible. Genetic variability studies based on class I genetic markers (erythrocyte antigens, blood serum proteins, MHC antigens) have recently been extended with analysis of class II markers (polymorphic restrictive fragments, minisatellite and microsatellite sequences) associated with polymorphic DNA sequences. Of the DNA markers microsatellite sequences have found the broadest application. At present, they form the most numerous group of genetic markers that are used to study the genetic structure and genetic variability of different sheep breeds.

Microsatellite sequences, also known as short tandem repeats (STRs), consist of tandemly repeated 1–6 nucleotide motifs dispersed throughout the genome and

have a high level of polymorphism compared with those of other molecular markers (Kemp et al., 1995; Tautz, 1989).

Microsatellite sequences are amplified with polymerase chain reaction (PCR). The analysis of amplified DNA fragments, originally performed with hybridization techniques where PCR products after electrophoresis were transferred on nylon filters and hybridized with radioactive labelled probes, was supplanted with analysis in automatic DNA sequencers (Ziegle et al., 1992; Levitt et al., 1994; Buchanan et al., 1994). Special applications were found for multiplex PCR, where the reaction mixture contains even a dozen or so starter pairs, enabling simultaneous duplication of more than ten loci. Amplification starters are fluorescently labelled with four dyes, making it possible to simultaneously detect more than ten microsatellite markers on a single lane of polyacrylamide gel (Tanaka et al., 1996, Luikart et al., 1999). Such electrophoretic separation is read by laser detectors, which eliminates the hybridization stage and enables direct reading of results and saving the data on a computer. Multiplexed fluorescent STR systems represent a fast, efficient and relatively inexpensive method of biodiversity analysis.

The aim of the study was to evaluate the usefulness of seven STR systems for biodiversity analysis of sheep.

Material and methods

A total of 73 Wrzosówka sheep were investigated. Microsatellite markers were identified in blood samples collected from the jugular vein into EDTA tubes.

Microsatellite polymorphism was analysed based on the BM757, BM6526, OarCP20, OarCP34, OarFCB128, OarFCB304 and OarHH35 markers recommended by FAO. Genomic DNA was isolated from 40 µl of blood using proteinase K, following the procedure of Kawasaki (1990). Based on the isolated DNA, the 7 microsatellite loci were amplified by the PCR. PCR reaction was performed on a GeneAmp 9600 thermocycler (Applera Biosystems).

Multiplex reactions were carried out using Multiplex PCR mix, containing 10× PCR buffer, dNTPmix (mixture of 4 deoxyribonucleotides, each of 1.25 mM), DNA polymerase AmpliTaq Gold (5U/µL) and 7 pairs of starter sequences.

One of each starter pairs was labelled at 5' end with a fluorescent dye and three fluorochromes were used: 5-FAM for the loci OarFCB128 and OarFCB304, TET for BM6526, OarCP20 and OarHH35, and HEX for BM757 and OarCP34.

Following an initial denaturation of genomic DNA for 15 min at 95°C, the reaction mixture was subjected to 31 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s, extension at 72°C for 60 s, and final extension at 72°C for 60 min. PCR products containing GeneScan 350-TAMRA size standard were separated by electrophoresis in a 4% denaturing polyacrylamide gel on a laser sequencer ABI PRISM 377 (Applied Biosystems). Results of the electrophoretic separation were analysed with GeneScan 2.1 software and the alleles were sized with Genotyper 2.0 software.

Results

DNA amplification products were obtained from all loci. The size range of alleles for each locus are presented in Table 1.

A total of 43 alleles were identified for the 7 microsatellite sequences studied. The number of alleles per locus varied from 5 for locus BM757, BM6526 and OarCP34 to 8 for OarFCB128 (Table 1).

Table 1. Marker characteristics in the studied populations of sheep

Marker	Length (bp)	Dye	Primer concentration (μM)	Allele No.
OarFCB128	99–127	FAM	0.3	8
OarFCB304	162–188	FAM	0.6	6
OarCP20	71–89	TET	0.3	7
OarHH35	119–133	TET	0.4	7
BM6526	162–170	TET	0.6	5
OarCP34	114–124	HEX	0.3	5
BM757	176–184	HEX	0.6	5

Discussion

The great number of DNA microsatellite sequences identified so far, characterized by a high degree of polymorphism and the development of automated methods for their analysis made it possible to investigate the genetic variability in sheep based on microsatellite markers (Arranz et al., 2001; Cerit et al., 2004; Tapio et al., 2005; Moioli et al., 2006; Radko et al., 2006; Nahas et al., 2008).

Microsatellite polymorphism is manifested as allelic length differences due to the different numbers of repeated units present in the alleles and is easily assayed by PCR amplification. The PCR-multiplex reaction, used to investigate the polymorphism of microsatellite sequences, minimizes the costs and duration of analysis (Hayen et al., 1997; Laird et al., 2005).

We selected seven microsatellite markers recommended by FAO to analyse biodiversity and examined their application in research on Wrzosówka sheep (ISAG/FAO Standing Committee, 2004). The criterion for marker combinations was based on the allele size difference between the largest allele of one marker and the smallest allele of another marker that will be neighbouring each other on the electrophoresis gel.

One of each starter pairs was labelled with a fluorescent dye: FAM, TET and HEX (Table 1). The application of a fluorescently labelled starter for amplification enhances the identification of PCR products with the Genescan software and the automatic genotyping of animals with the Genotyper software (Alford et al., 1994; Mansfield et al., 1994; Tanaka et al., 1996).

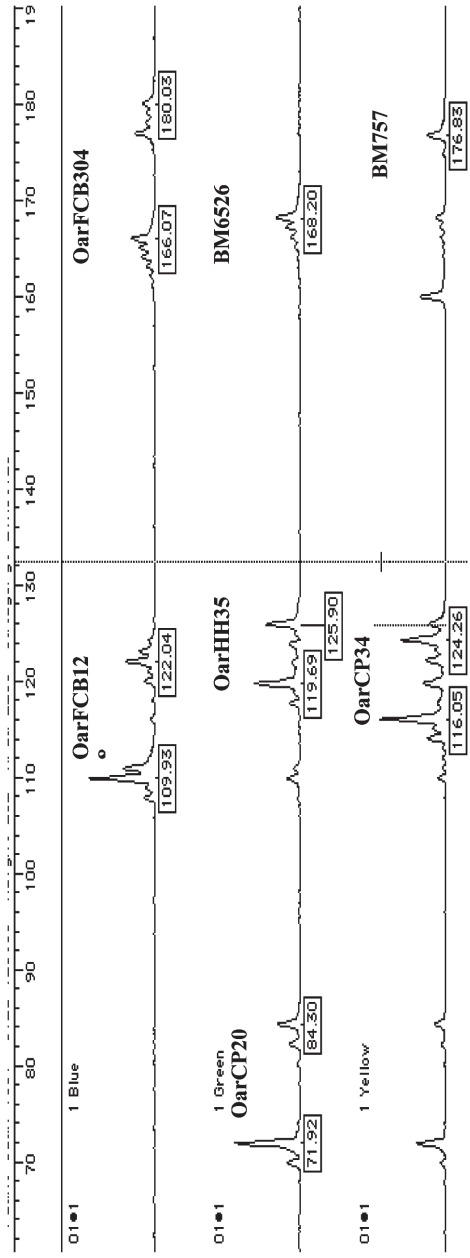

Figure 1. DNA profiles of the investigated panel of STR loci amplified in multiplex reaction

The multiplex PCR amplifications were performed to analyse all loci together. The annealing temperature and primer concentration was in most cases the key for successful amplification at all loci. In general, adjustment of the primer concentration was the main factor in obtaining well-readable DNA profiles for amplified loci in PCR multiplex conditions. First, markers were individually amplified to choose the optimal annealing temperature of primers to be later combined in the multiplex reaction. Annealing was performed at 50°C, 53°C, 56°C, 59°C and 62°C for all microsatellites. Finally, the microsatellites were simultaneously amplified in one polymerase chain reaction containing the primer concentrations for each marker. The primer concentrations are presented in Table 1. Optimal annealing temperature was 59°C.

The lengths of amplified fragments were analysed by automated DNA sizing technology, using a laser ABI 377 DNA sequencer (Ziegle et al., 1992).

In the animals analysed, DNA amplification products were obtained from all loci. The DNA profiles of the investigated microsatellite loci are presented in Figure 1.

The results suggest that the 7-plex fluorescent STR-PCR system can be applied for structure population analysis in sheep.

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Ocena przydatności polimorfizmu 7 markerów mikrosatelitarnych DNA do analizy zróżnicowania genetycznego owiec

STRESZCZENIE

Celem badań było określenie polimorfizmu wybranych sekwencji mikrosatelitarnych u owiec rasy wrzosówka, poprzez zastosowaniu reakcji PCR-multiplex. Do badań wybrano 7 markerów zalecanych przez FAO do oceny bioróżnorodności: BM757, BM6526, OarCP20, OarCP34, OarFCB128, OarFCB304, OarHH35. Jeden z każdej pary starterów znakowany był fluorescencyjnym barwnikiem: FAM, TET i HEX. Głównymi czynnikami wpływającymi na uzyskanie produktów PCR było ustalenie temperatury przyłączania sekwencji starterowych do matrycy (58°C) oraz koncentracji starterów w mieszaninie reakcyjnej (od 0,3 do 0,6μM). Długości otrzymanych fragmentów DNA określano techniką automatycznej analizy fragmentów w sekwenatorze ABI377. U badanych osobników otrzymano produkty PCR we wszystkich analizowanych loci. Liczba otrzymanych alleli w poszczególnych loci wyniosła od 5 w loci BM757, BM6526 i OarCP34 do 8 w locus OarFCB128. Otrzymane wyniki wskazują na możliwość zastosowania opisanej reakcji 7plex-PCR i automatycznej analizy wielkości fragmentów do badania zróżnicowania genetycznego owiec.