SELECTION OF OPTIMAL ISSR-MARKER SYSTEMS FOR HORSES IN THE UKRAINE

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Abstract

Seven populations of horses (*Arabian, Orlov Trotter, Trakehner, Thoroughbred, Novooleksandrivska draughtbreed, Ukrainianracebreed,* and *Przewalski*) were studied to identify the most informative Inter-Simple Sequence Repeat (ISSR) marker systems. The number of amplicons and polymorphic locivaried considerably depending on the microsatellite motifused as a primer. In the first stage of our research we screened 10 marker systems on the basis of tri-nucleotide primers with different sequences and anchored nucleotides on the 3' end. Based upon visual inspection of the clarity of the bands and potential for a high degree of polymorphisms, 4 of the 10 markers were selected for further evaluation. These 4 markers [$(AGC)_6G$, $(ACC)_6G$, $(GTG)_6A$, $(AGC)_6C$] were then used for the evaluation of variation within and across breeds by pooling DNA from individual horses for a specific breed. The $(ACC)_6G$ primers were associated with the most loci (24) and most polymorphic loci (19). The $(AGC)_6G$ primers resulted in the second highest number of loci (19) and polymorphic loci (15). These markers can be used for determination of a genetic distance between breeds and searching for phylogenetic connections. Use of these markers will also allow for an expansion of the pool of information about genetic variations among horse breeds.

Key words: horse, ISSR, marker system, marker index.

INTRODUCTION

Monitoring of genetic polymorphisms within populations is an important component of breed maintenance and reproductive programs in many agricultural species, including horses. Characterization of the population genetic structure can become the first step toward breed preservation and restoration, and contributes to advancing breeding programs (Iwanczyk, et al., 2006).This is particularly important in countries such as the Ukraine, where a method of validating horse identification through genetic testing has not been resolved. The between-breed differences in phenotypic and morphophysiological traits are readily discernible; however, the gene pool differences among the breeds still remain poorly investigated (Stolpovskii, 2010).

Formerly, testing blood groups and plasma protein was used to verify parentage and breed composition. The development of DNA-technologies has allowed for the development of more advanced methods of identification. Genotyping of animals by DNA microsatellite lociis widespread. However, these test systems can be not always appliedfor the analysis of aboriginal breeds due to poor investigation of their genomes with respect to the microsatellite alleles and their frequencies. The high cost of theequipment and kits also makes their use problematic formass population studies. Nevertheless, other DNA-genetic methods of identification such as Inter-Simple Sequence Repeat (ISSR) analysis are available and are more adaptable to the Ukrainian equine industry. This technology is more easily adapted to the study of unique breeds where nucleotide sequences are unknown and less expensive than Simple Sequence Repeat (SSR)-PCR techniques used for microsatellite genotyping. The ISSR-PCR method of amplifying inter microsatellites fragments of DNA allows estimation of genomic variability between two inverted SSR-loci (Zietkiewicze et al., 1994; Bornet et al., 2002; Bardukov, 2010; Metlitzka, 2012). ISSR-PCR is characterized by reproducibility and can be effectively used to examine genetic variation within and across breeds, and enable authentication of species and populations when compared with other methods of multiloci profiling (Stolpovskii, 2010; Feofilov, 2011).

Analysis of the spectra of amplification products of the regions between the inverted repeats of microsatellite loci was used for identification of interspecific and interbreed differences in cattle (subfamily Bovinae) (Azari et al., 2006; Stolpovskii et al., 2011), yaks (Azari et al., 2006), sheep (Stolpovskii, 2010), deer (Kol and Lazebnyi, 2006) and other species.

ISSR analysis has also been applied for investigation of populations of aboriginal horse breeds. Di-nucleotide based ISSR markers used for analysis of horse populations in other research have demonstrated low effectiveness because of a low number of amplified fragments (Kol and Lazebnyi, 2006). However, the use of tri-nucleotide primers for has resulted in patterns with a large number of clear bands (Kuhl, 1993; Voronkova, 2011).

The objective of our research was to identify the most informative ISSR marker systemsusing tri-nucleotide primers for verification of horses in the Ukraine. Secondly, we wanted to select the polymorphic ISSR-marker systems that would best enable the study of genetic variation of horse populations (Equus caballus and Equus przewalski).

MATERIALS AND METHODS

Biological material (blood or hair follicules) were retrieved from 170 representatives of 7 horse populations (Arabian (n=16), Orlov Trotter (n=32), Trakehner (n=10), Thoroughbred (n=32), Novooleksandrivska draught (n=32), native Ukrainian racehorses (n=32), and Przewalski (n=16)). Genomic DNA was extracted per manufacturer instruction from the horses' hair follicles and blood using a DNA-sorb kit (AmpliSens, Russia, Moscow) with modifications to incubation temperature and chemical concentrations according to Carter and Milton (1993).

DNA concentrations and quality were determined with a NanoDrop (ThermoFisherScientific, Germany). Amplification was conducted using the Thermal Cycle amplifier (DNA-Technology, Moscow, Russia) and the following thermal cycles: initial denaturizing for 4 min at 94° C; 32 cycles at 94 °C for 30 sec, 58 °C for 30 sec, 2 min at 72 °C; and 5 min at 72 °C.

The reactionary mixture by volume of 20 µl contained: 67 mM Tris - HCl (pH 8.8), 17 mM $(NH_4)_2SO_4$, 0.01% Tween - 20, 0.2 mM dNTP, 1.0 unit of Tag-polymerase, 60 ng of DNA, 1.5 mM $MgCl_2$ and 0.5 mM of primer. The optimal concentration of each component was selected experimentally.

The first stage of our research included a 10 marker system on the basis of tri-nucleotide primers with different sequences and anchored nucleotides on the 3' end (Table 1). For this screening, we pooled DNA from all breeds in order to identify ISSR primers that showed a large degree of variation. Based upon visual inspection of the clarity of the bands and potential for a high degree of polymorphisms, 4 of the 10 markers were selected for further evaluation. These 4 markers were then used for the evaluation of variation within and across breeds by pooling DNA from individual horses for a specific breed. The four selected primer sets were then analyzed three times for each breed.

The electrophoretic division of amplification products was conducted with a 1.5% agarose gel using 0.5 × TBE- buffer at 100 V for 80 minutes. The electrophoresis gel was processed by bromide ethydium (0.5 µg/ml) and visualized under UF-rays and pictures with a digital Panasonic DMC-FS42 camera. For determination of molecular mass, the Gene Ruler 100 bp (Fermentas, Lithuania, Riga) was used. Quantity One (Bio-Rad Laboratories, USA) software was used to identify lanes and define, quantify, and match bands. The Volume Tools option of Quantity One was used to measure and compare the bands.

Results from the electrophoretic division of PCR productswas converted to a binary form: 1 is presence, 0 is absence of a band (fragment) in the properposition on the track. Fragments that were visualizedin at least two different gels at the same location were considered identical. The matrices obtained were used for further analysis by GenAlEx 6 (Peacall, 2006). Diagnostic measures included the number of loci (nlop), number of polymorphic loci (np), percentage of polymorphic loci (Na), number of effective alleles [Ne = 1/(p^2 + q^2)], and expected heterozygosity [H_e = 2 * p * q].

These measures were then used to determine the Marker Index (MI) (Metlitzka, 2012) as:

MI = E*H_{av},

where H_{av} = H_e/np; and E = nlop*np.

RESULTS AND DISCUSSION

The primers for the first stage of research (Table 1) were selected because they had demonstrated clear band patterns with high reproducibility and specificity in other animal populations (Berezovskaya, 2002; Glazko, 2001). The ISSR-PCR amplification patterns obtained for these primers in our population are shown in Figure 1 and our evaluation of each primer set is in Table 2. The S3 and S8 marker-systems were not effective for the identification of genetic variants as there were no PCR products. The S1 and S5 primer systems were also ineffective because they produced diffuse spectrums without clear, discrete bands. The remaining primers (S2, S4, S6, S7, S9, S10) resulted in clear areas of amplification. Of these, S2, S6, S9, and S10 provided the most clearly defined bands and were selected for further evaluation.

Table 1. Description of the marker systems evaluated

No	Primer	Repeats	Nucleotide sequence 5→3
S1	$(CTC)_6A$	$(CTC)_6$	CTC CTC CTC CTC CTC CTC A
S2	$(AGC)_6G$	$(AGC)_6$	AGC AGC AGC AGC AGC AGC G
S3	$(TCG)_6G$	$(TCG)_6$	TCG TCG TCG TCG TCG TCG G
S4	$(CTC)_6C$	$(CTC)_6$	CTC CTC CTC CTC CTC CTC C
S5	$(GAG)_6G$	$(GAG)_6$	GAG GAG GAG GAG GAG GAG G
S6	$(GTG)_6A$	$(GTG)_6$	GTG GTG GTG GTG GTG GTG A
S7	$(CCA)_6G$	$(CCA)_6$	CCA CCA CCA CCA CCA CCA G
S8	$(GCT)_6A$	$(GCT)_6$	GCT GCT GCT GCT GCT GCT A
S9	$(AGC)_6C$	$(AGC)_6$	AGC AGC AGC AGC AGC AGC C
S10	$(ACC)_6G$	$(ACC)_6$	ACC ACC ACC ACC ACC ACC G

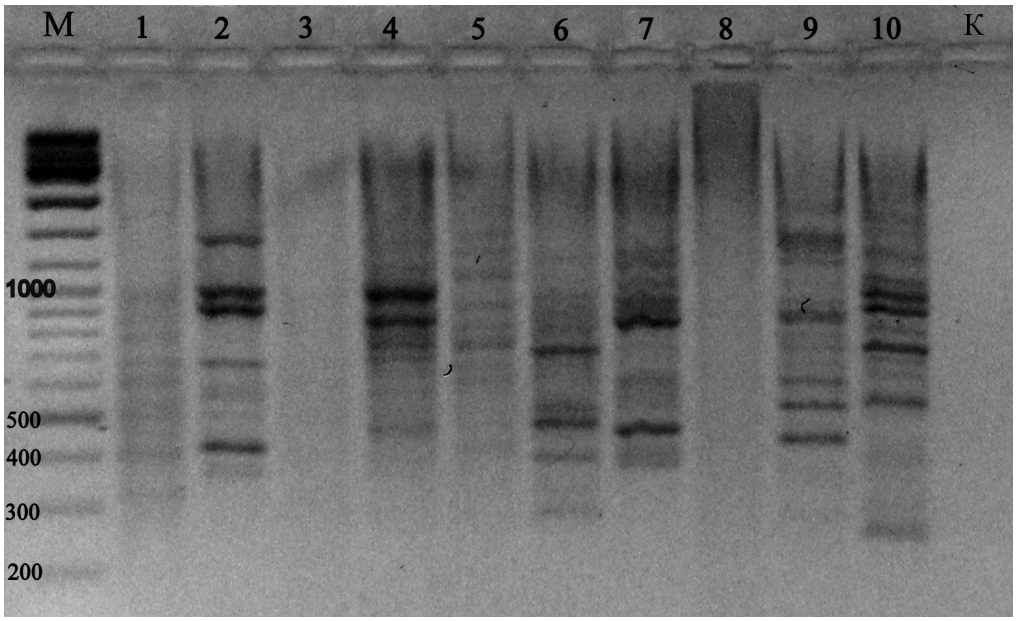

Fig. 1. *Screening ISSR-primers with tri-nucleotidesequenses: M – marker of molecular size (GeneRuler DNA Ladder Mix, Fermentas), 1 - $(CTC)_6A$, 2 - $(AGC)_6G$, 3 - $(TCG)_6G$, 4 - $(CTC)_6C$, 5 - $(GAG)_6G$, 6 - $(GTG)_6A$, 7 - $(CCA)_6G$, 8 - $(GCT)_6A$, 9 - $(AGC)_6C$, 10 - $(ACC)_6G$*

The amplification patterns obtained in the second stage of research for the 4 selected markers are demonstrated in Figures 2 and 3. For the data analysis we used only clear PCR-loci that were reproduced in all 3 independent replicates. Diffuse, low-molecular fragments with lengths between 80-150 bp were not reproduced consistently across replicates and were not considered. There were no fragments identified that exceeded 1500 bp. Every product of amplification was examined as a separate locus.

Descriptive statistics for each primer set are reported in Table 3. Across the 4 primer sets were obtained 74 products of amplification and 54 of these (72.97%) were polymorphic. The total number of PCR products rangedfrom14 (S6) to 24 (S10). The S9 primers resulted in the lowest number of polymorphicloci (9), where S10 resulted in the highest (19).

The amplicon spectrum was stratified into heavy fragments (more than 1000 bp), midrange (500 to 1000 bp) and low-molecular weight fragments (300 to 500 bp).

The S10 primers were associated with the most loci and most polymorphic loci. The length of S10 PCR-products was in a range of 260 to 1430 bp. The majority of fragments were midrange (63.16%), and 36.84% were low-molecular weight fragments. The S2 primers resulted in the second highest number of loci and polymorphic loci. S2 amplicons ranged in length from 270 to 1400 bp. The majority of fragments were midrange (83.33%), and 4 (16.67%) were shorter than 500 bp.

Table 2. The number of amplicons identified for each primer set and their visual band quality

No	Primer	Amplicons (n)	Band quality
S1	$(CTC)_6A$	9	Diffuse
S2	$(AGC)_6G$	9	Clear
S3	$(TCG)_6G$	-	none
S4	$(CTC)_6C$	7	clear
S5	$(GAG)_6G$	10	Diffuse
S6	$(GTG)_6A$	7	Clear
S7	$(CCA)_6G$	5	Clear
S8	$(GCT)_6A$	-	None
S9	$(AGC)_6C$	11	Clear
S10	$(ACC)_6G$	11	Clear

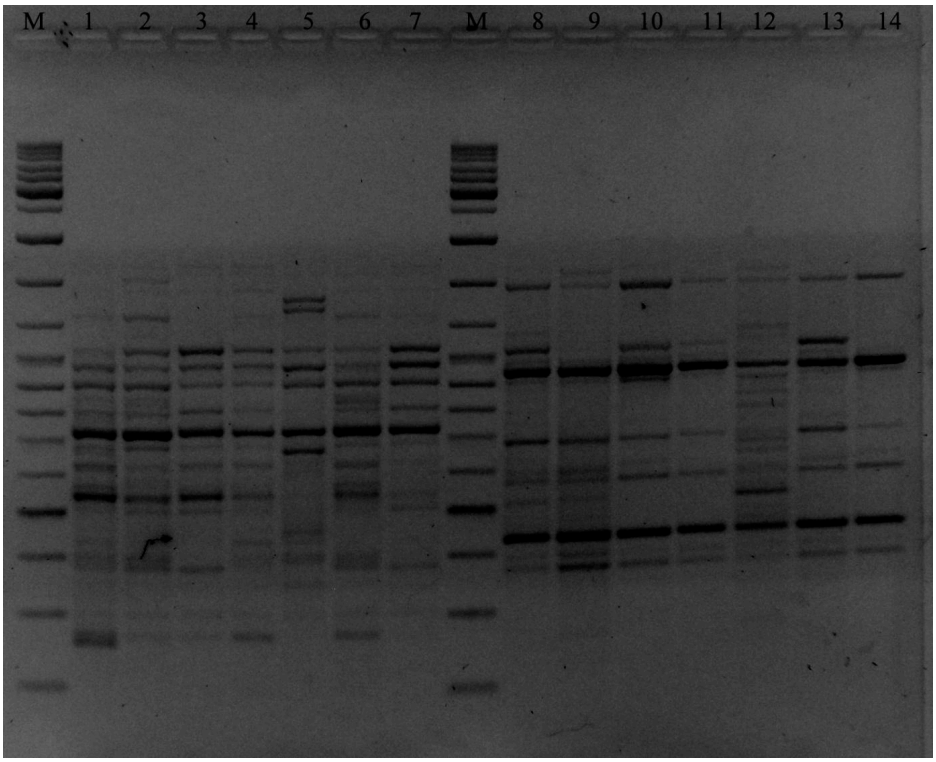

Fig. 2. *ISSR-PCR profile generated by primers $(ACC)_6G$ (lanes 1 through 7) and $(AGC)_6G$ (lanes 8 through 14) of different breeds of horses; Arabian (lanes 1 and 8), Przewalski horse (lanes 2 and 9), Orlov Trotter (lanes 3 and 10), Trakehner (lanes 4 and 11), 5, 12 Novooleksandrivska (lanes 5 and 12), 6, 13 – Thoroughbred (lanes 6 and 13), native Ukrainian race horses (lanes 7 and 14); M - marker of molecular sizes (GeneRuler DNA Ladder Mix, Fermentas*

The S6 (11) and S9 (9) marker systems had fewer polymorphic loci than S2 and S10, and S9 had the lowest percentage of polymorphic loci (46.67%) of the primers evaluated. Amplicon lengths for S6 (300 to 1120 bp) and S9 (420 to 1450 bp) and the proportion of midrange amplicons (78.57% and 88.24%, respectively) were similar to S2 and S10.

Based upon the number of loci, degree of polymorphic loci, and expected heterozygosity, the S10 primers (5.7) and S2 primers (3.9) were associated with the highest MI. For that reason, the S10 and S2 primers were considered the most suitable to determine within and across breed genomic variation for Ukrainian horses.

Table 3. The number of loci present (nlop), number of polymorphic loci (np), the percentage of polymorphic loci, and marker index (MI)

No.	Primer	nlop	np	Polymorphic (%)	MI
S2	$(AGC)_6G$	19	15	76.47	3.9
S6	$(GTG)_6A$	14	11	78.57	3.5
S9	$(AGC)_6C$	17	9	46.67	2.5
S10	$(ACC)_6G$	24	19	79.17	5.7
	Σ	74	54	72.97	-

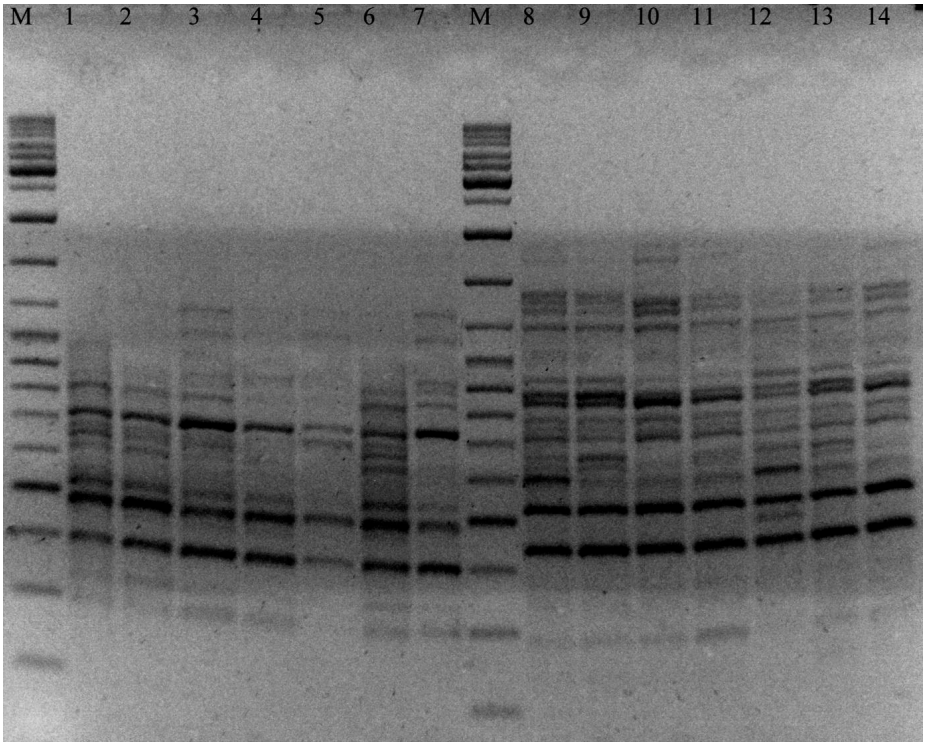

Fig. 3. *ISSR-PCR profile generated by primers $(GTG)_6A$ (lanes 1 through 7) and $(AGC)_6C$ (lanes 8 through 14) of different breeds of horses; Arabian (lanes 1 and 8), Przewalski horse (lanes 2 and 9), Orlov Trotter (lanes 3 and 10), Trakehner (lanes 4 and 11), 5, 12 Novooleksandrivska (lanes 5 and 12), 6, 13 – Thoroughbred (lanes 6 and 13), native Ukrainian race horses (lanes 7 and 14); M - marker of molecular sizes (GeneRuler DNA Ladder Mix, Fermentas*

ISSR DNA markers have been applied to the determination of polymorphism and genetic variety in plant-growing (Raina et al., 2001; Bento et al., 2008), but they have not been as broadly applied in livestock experiments. According to our results, this type of marker is useful for determination of polymorphism and genetic distance in horse breeding.

The lack of amplification for the S3 and S8 marker-systems may indicate an absence of these microsatellites loci with six repetitions in the equine genome. While PCR effectively amplified loci with S1 and S5 primers, there may have been insufficient distance (<3kb) between loci to produce clear bands resulting in diffuse spectrums without clear, discrete bands. Absence of discrete bands during the electrophoretic division may also indicate an abundance of these microsatellites in the equine genome.

To evaluate the marker system's informativeness, we analyzed MI which is associated with the level of heterozygosity. Voronkova (2011) compared alternative marker systems with a Polymorphism Information Content (PIC) index and the highest PIC scores were obtained for GAG and ACC markers. Our results also identified ACC marker as being highly informative for across-breed horse typing. However, we did not evaluate GAG for the second stage of our research due to the observation of diffuse spectrums without clear discrete bands at the first stage of our research.

In general, all of the marker systems tested in the second stage could be useful for interbreed and interspecies research of horses. The most accurate genetic information will be obtained as the number of marker systems that are used increases. However, cost considerations limit the number of systems that can be practically tested in many research settings. The price of ISSR-typing per 100 horsesis $228 per primer set in the Ukraine.

CONCLUSIONS

1. Given the need to minimize testing costs in the current Ukrainian economic environment plus the high informativeness of ISSR-S2 $(AGC)_6G$) and S10 $(ACC)_6G$ marker systems, it is recommended that these be used in further research with these horse breeds. Use of these markers also will allow for an expansion of the pool of information about the genetic variety of horse breeds due to the inclusion of unique breeds with unique alleles in this research.

2. These markers can be used for determination of genetic distance between breeds and searching phylogenetic connections. Our future research with ISSR typing will be expanded to determine the unique alleles for more horse breeds and the genetic distance between breeds according to ISSR genotype.

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