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The Malopolski Horse Stallions: Genetic Diversity Estimated on the Basis of Microsatellite DNA and Class I Markers

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Original article

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The Malopolski horse (*Equus caballus*), a halfbred warmblood breed influenced by Thoroughbred horse, was created in southern and south-eastern Poland. Four sire lines were mostly involved in Malopolski horse breeding. Today, Malopolski horse is in the genetic resources conservation programme. The aim of our study was to evaluate the genetic diversity of Malopolski horse stallions (251 individuals). We used two types of markers: a recommended panel of 17 STRs (microsatellites) and class I markers (polymorphic blood proteins and enzymes). We also present some historical facts about the origins of the Malopolski horse. We estimated observed and expected heterozygosities (H_o and H_e), the mean inbreeding index value (F_{IS}), the polymorphic information content (PIC), power of discrimination (PD) and probability of parentage exclusion. The studied population of Malopolski stallions exhibited high diversity of genotypes and alleles. We identified 33 phenogroups among blood cell antigens, inherited in seven system groups. The mean number of STR alleles per locus was 7.706 ± 0.460. We confirmed the usefulness of both sets of markers for parentage analysis of the Malopolski horse breed.

Key words: Microsatellite markers, STR, parentage testing, horse, Malopolski horse, Thoroughbred horse, class I marker.

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The Malopolski horse (*Equus caballus*) is a Polish halfbred warmblood breed strongly influenced by Thoroughbred horse (SONDIJ 2011), probably the best-known horse breeds in the world (ZABEK 2008). The Malopolski horse breed consists mostly of four sire lines: Furioso, Gidran, Nonius and Przedswit (SONDIJ 2011; ZABEK *et al*. 2006). The sire lines originated from former Austro-Hungarian horses and had an impact not only on creating the Polish breed. Lines of Furioso, Gidran and Przedswit were engaged inter alia in Czech autochthonous breeds (PUTNOVA *et al*. 2018); lines except Przedswit had a hand in creating breeds including Furioso breed (BUROCZIOVA *et al*. 2008), Gidran breed (MIHOK *et al*. 2005; SZISZKOSZ *et al*. 2016) or Nonius breed (MORAVCÍKOVA *et al*. 2016) (history of sire lines in Suppl. Information).

The Malopolski horse was created over the centuries (Suppl. Information) and now is priceless in Polish material culture. Registers for Malopolski horse have been kept since 1963. Since the 19th century, due to the rise of mechanisation in agriculture, the Malopolski horse has been used more often in sport and leisure riding than in transportation or horse-powered machines (ZABEK *et al*.

2006). Its population has fallen by half since 2005 (POLISH HORSE BREEDERS ASSOCIATION 2018). In 2005, the genetic resources conservation programme was initiated for the Malopolski horse. Today, the Malopolski horse is a one of seven Polish horse breeds in the genetic resources conservation programme (PASTERNAK 2016). Modern Malopolski horse breeding focuses on saddle horses for sport and recreation. As an outstanding Polish horse breed, it should be preserved by comprehensive zootechnic work and further improvement of functional features. The balance between breeding (utility traits – sports competition results) and genetic resources conservation programmes (to preserve unique gene combinations and related functional traits) is extremely important (SONDIJ 2011).

To preserve the breed, genetic variation should be monitored. Valuable genetic information can be gained by using various types of markers (mostly STRs for horse parentage verification or recommended by FAO) (FAO 2011; FORNAL *et al*. 2014; STACHURSKA *et al*. 2014).

We investigated molecular markers to evaluate the genetic diversity of Malopolski horse stallions – offspring of all sire lines described above. We used the recommended panel of 17 microsatellite loci (STRs) and polymorphic blood proteins and enzymes (class I markers).

Material and Methods

Genetic variation was examined in 251 Malopolski horse stallions born between 2003 and 2013 in Poland (various studs, randomly selected). Analysis of polymorphic genetic markers was conducted with markers class I and II; we used blood samples for routine horse parentage testing.

Microsatellite markers

DNA profiles were specified using 17 microsatellite loci recommended by the International Society for Animal Genetics (ISAG) for horse parentage verification: 12 markers from the ISAG core panel: AHT4, AHT5, ASB2, HMS2, HMS3, HMS6, HMS7, HTG10, HTG4, VHL20, ASB17, ASB23; five markers from the ISAG additional panel: HTG6, HTG7, CA425, HMS1 and LEX3.

Each sample of DNA was isolated from 300 µl of peripheral blood using Wizard kit (Promega-Wizard Genomic DNA Purification Kit) or Sherlock AX (A&A Biotechnology) according to the instructions of the manufacturer. DNA was amplified in one multiplex reaction (17 loci) using Equine Genotypes Panel 1.1 Kit (ThermoFisher Scientific) with positive and negative DNA controls. STRs were amplified according to the manufacturer's protocols, with 17 primer pairs labelled with five fluorescent dyes. PCR products were analysed in a capillary electrophoresis system on an 3130xl Genetic Analyser (Applied Biosystems). The separation of products was conducted with GeneScan-500 LIZ Size Standard (Applied Biosystems) in each sample well, enabling automated DNA fragment analysis. Next, the data were processed in GeneMapper 4.0 software (Applied Biosystems). STR markers were standardised following ISAG recommendations. Our method is regularly checked and validated through participation in ISAG horse parentage comparison tests.

We analysed the results with GenAlEx6 (PEAKALL & SMOUSE 2006) and GenePop software (RAYMOND & ROUSSET 1995) to estimate observed and the expected heterozygosities (H_o and H_e), the mean inbreeding index value (F_{IS}), and the polymorphic information content (PIC). Other coefficients were estimated with custom-made software based on a method suggested by HUSTON (1998): power of discrimination (PD) and power of exclusion (PE). The frequency of alleles detected was used to calculate the probability of parentage exclusion for each locus, when the genotypes of one and both parents are known (PE_1 and PE_2) and the combined probability of parentage exclusion (CPE) for 16 loci together (JAMIESON & TAYLOR 1997). THE 17th locus LEX3 was excluded from the analysis due to X chromosome localization.

Markers class I

During the study, erythrocyte antigens were identified by serological and haemolytic tests with 35 reagents standardised by ISAG tests. Polymorphic forms of serum proteins were also identified. We used the method described by STORMONT & SUZUKI (1964) and STORMONT *et al*. (1964) to identify erythrocyte antigens. Alleles in seven system groups (loci of the EAA, EAC, EAD, EAK, EAP, EAQ and EAU) were estimated directly from the genotypes. We used the method of electrophoretic separation on polyacrylamide gels according to JUNEJA *et al*. (1978) to identify the polymorphic forms of plasma proteins. Frequencies of albumin alleles, protein GC, esterase, protein XK and transferrin were calculated based on identified phenotypes. The average theoretical degree of heterozygosity in the material was computed from the NEI & ROYCHOUDHURY (1974) formula. The rank of polymorphism PIC was calculated according to the formula defined by BOTSTEIN *et al*. (1980).

Results and Discussion

Microsatellite markers

All 17 microsatellite markers were obtained and typed. LEX3 loci was excluded from the analyses due to its localization on the X chromosome. The mean number of alleles per locus was 7.706 ± 0.460, the number of effective alleles was 4.077 ± 0.321 and the frequency of alleles less than or equal to 5% was 4.471 ± 0.273. We detected no private alleles.

The number of alleles per locus was generally quite similar to results obtained by MIHOK *et al*. (2005) for twelve STRs for Gidran and Nonius breeds. Based on the detected alleles we estimated the observed and the expected heterozygosities (H_o and H_e), the mean inbreeding index value (F_{IS}) and the polymorphic information content (PIC) (Table 1). The data showed no inbreeding in the tested population with F_{IS} from -0.049 (HTG6) to 0.084 (CA425). ZABEK *et al*. (2006) also calculated that the F_{IS} coefficent for Malopolski horse was close to zero. Other estimated coefficients included mean PIC value of 0.687; H_o and H_e values were similar (0.729 and 0.726 respectively). H_o estimated by us was slightly higher than H_o calculated by ZABEK *et al*. (2006) (mean H_o for subpopulations of Malopolski horse from 0.671 to 0.719), similar to H_o obtained by BERBER *et al*. (2014) for Thoroughbred and Arabian (related to Malopolski horse), and similar to Polish Arabian obtained by GLAZEWSKA *et al*. (2018). Mean H_e was similar to the value of this coefficient estimated for Furioso by KASARDA *et al*. (2018).

Among core panel loci, nine loci were highly polymorphic. Two loci: HMS2 and HTG4 had H_e, PIC, H_o values below 0.7. Four additional microsatellites (marked with asterisks in Table 1) had generally lower values of those coefficients versus the core panel.

We estimated the power of discrimination (PD) for each marker (data not shown) and for all markers together, so the cumulative power of discrimination (PD_c) was 0.999989. The combined probability of exclusion (PE) was calculated when only one parental genotype is known (PE_1) or for both parental genotypes (PE_2) (HUSTON 1998) (Table 2). The values of coefficients were similar to those in the literature (FORNAL *et al*. 2014; NIEMCZEWSKI & ZURKOWSKI 2000). The cumulative power of exclusion (CPE) was 0.999504 for CPE_1 and 0.999998 for CPE_2.We assessed the lowest PE coefficient values for single loci for HTG7, HMS1 and HTG4. Nonetheless, the cumulative power of exclusion for the whole set is very high (99.999%).

Markers class I

We identified 33 phenogroups in Malopolski stallion blood cell antigens, inherited in seven system groups (Suppl. Table 3S). The highest diver-

Table 1

The polymorphic information content (PIC), the observed (H_o) and expected heterozygosities (H_e) inbreeding index value (F_{IS}), for STRs; * – additional microsatellites markers in STRs set

Locus	PIC	H_o	H_e	F_{IS}
AHT4	0.777	0.839	0.804	-0.044
AHT5	0.719	0.701	0.755	0.072
ASB2	0.755	0.793	0.786	-0.010
HMS2	0.647	0.690	0.675	-0.021
HMS3	0.782	0.785	0.807	0.027
HMS6	0.671	0.743	0.714	-0.041
HMS7	0.760	0.766	0.791	0.031
HTG10	0.837	0.893	0.854	-0.045
HTG4	0.520	0.571	0.587	0.028
HTG6*	0.636	0.728	0.694	-0.049
HTG7*	0.498	0.575	0.549	-0.047
VHL20	0.728	0.793	0.766	-0.036
ASB17	0.744	0.801	0.773	-0.036
ASB23	0.758	0.774	0.786	0.016
CA425*	0.654	0.625	0.682	0.084
HMS1*	0.515	0.594	0.590	-0.007
Mean	0.687	0.729	0.726	

Table 2

Probability of exclusion for sixteen STRs

Locus	PE_1	PE_2
AHT4	0.441	0.673
AHT5	0.360	0.430
ASB2	0.409	0.586
HMS2	0.286	0.412
HMS3	0.448	0.572
HMS6	0.305	0.498
HMS7	0.412	0.538
HTG10	0.539	0.781
HTG4	0.186	0.257
HTG6	0.265	0.473
HTG7	0.156	0.262
VHL20	0.370	0.586
ASB17	0.396	0.600
ASB23	0.412	0.552
CA425	0.293	0.321
HMS1	0.183	0.284
CPE	0.996	0.999

sity occurred in the D system (12 alleles). A characteristic feature of the studied population of Malopolski stallion is high occurrence of alleles adf (0.650), Ca (0.956), dkl (0.408), P (0.578) and U (0.822). Frequency analysis of protein alleles (Suppl. Table 4S) revealed that the studied population is highly diversified (33 phenotypes and 16 electrophoretic variants). We found quite low occurrence of ALA (0.336), GCS (0.058) and ESF (0.128). The most polymorphic was transferrin with 20 different phenotypes. The most frequent allele was TFF2 (0.404) and the least frequent allele was TFR (0.022). All results were comparable to results obtained by NOGAJ & NOGAJ (2000), including the high frequency of the dkl allele. According to NOGAJ *et al*. (2003) a high frequency of this allele often occurred in Polish halfbred warmblood horses such as Malopolski or Wielkopolski horse and other breeds including Hanoverian or Holsteiner.

Mean H_o and PIC for class I markers were 0.411 and 0.385, respectively (Suppl. Table 5S). Mean H_o was similar to heterozygosity observed by NOGAJ *et al*. (2003) for Malopolski horse (0.466) and also for a randomly selected collection obtained by STACHURSKA *et al*. (2014) (0.371). Polymorphic information content per locus was quite similar to that observed by NOGAJ *et al*. (2013).

According to the literature, PE calculated for both types of markers indicated higher accuracy of microsatellite markers versus class I markers. PE calculated on the basis of seven blood group and 11 polymorphic blood proteins and enzymes was 97.30% and on the basis of 17 STRs was about 99.99%. Higher PE value was one of the reasons for replacing class I markers with STRs in parentage testing (FORNAL *et al*. 2014; NIEMCZEWSKI & ZURKOWSKI 2000).

Conclusions

The studied population of Malopolski stallions is characterised by a high diversity of genotypes and alleles. There is no danger of inbreeding and the population seems genetically stable. Such diversity is a preferred feature in parentage verification. Consequently, there is a high probability of finding errors in the individual's pedigree. The observed polymorphism also confirmed that both sets of markers were appropriate for Malopolski horse breed parentage testing and genetic diversity estimation.

Analysis of core microsatellite markers (12) is sufficient to assess genetic diversity in this breed. However, using the whole recommended panel of 16 STRs (17th locus LEX3 was excluded from the analysis due to X chromosome localization) provides the most comprehensive information. Our analysis confirms this.

Malopolski horse has been created over the centuries. Today the breed is priceless in Polish material culture and is also an important part of Austro-Hungarian legacy due to its origins. Because of its declining population, the breed is included in the genetic resources conservation programme. The genetic variability of the breed should be constantly monitored. The markers used for parentage control are a natural choice for such studies.

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Author Contributions

Research concept and design: A.F., A.R.; Collection and/or assembly of data: A.F., J.N., K.Z-D.; Data analysis and interpretation: A.F., A.R., J.N.,

K.Z-D., A.P-K.; Writing the article: .A.F., A.R., J.N., K.Z-D., A.P-K.; Critical revision of the article: A.F., A.R., J.N., K.Z-D., A.P-K.; Final approval of article: A.F., A.R., J.N., K.Z-D., A.P-K.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary Material to this article can be found online at:
http://www.isez.pan.krakow.pl/en/folia-biologica.html

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