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Polymorphism in introns 5 and 6 of the *ACTA1* gene in various Polish horse breeds

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Abstract: The aim of this study was to find polymorphisms in the actin alpha 1 gene (*ACTA1*) in horses belonging to different breeds and utility types and to compare them. The genomic region of the actin alpha 1 gene was positively selected in purebred horses. By sequencing part of the *ACTA1* gene, 2 polymorphic sites located in intron 5 (G > T) and intron 6 (G > C) were found. The polymerase chain reaction-restriction fragment length polymorphism and the amplification created restriction site-polymerase chain reaction methods were applied to screen these polymorphisms. An analysis of the genotype and allele frequencies showed differences depending on the breed and utility type. Other genetic parameters such as heterozygosity and gene diversity revealed similar relationships. For some breeds, deviation from genetic equilibrium was observed. Haplotype analysis showed that 2 of 4 haplotypes were common to all breeds, 1 haplotype was absent in primitive horses, and 1 haplotype was observed only in Purebred Arabian horses and Małopolski horses (a breed that is known to have Arabian ancestors). It seems reasonable to conclude that distribution of the haplotypes of the *ACTA1* gene among the populations studied may be the result of long-standing selection for physical performance.

Key words: Horse, utility type, single nucleotide polymorphism, *ACTA1* gene

1. Introduction

While the physiological basis of physical ability in horses is well known, its genetic background is less understood, and there is an increased interest in this area of research. Polymorphism of the myostatin gene (*MSTN*) may be an example. Investigations have shown that it is associated with racing performance in Thoroughbred horses (1,2).

Candidate genes for physical performance refer to muscle strength and composition, muscle metabolism and exercise intolerance, hemodynamic and aerobic metabolism capacity, tendon and ligament physiology, and mind and motivation. One of the approaches for detecting candidate genes is screening for genomic regions that have been under selection pressure for athletic performance and analyzing the genes within these regions (3).

As a result of a genome scan for positive selection in Thoroughbreds, several regions with candidate genes were determined. The second region with highest interpopulation differentiation value (F_{st}) and relatively low diversity in Thoroughbreds was located on chromosome 1. It contains the microsatellite marker *TKY01*, which is situated 2.8 Mbp from the *ACTA1* gene and which has been proposed as a candidate marker for an athletic-performance gene in horses (4). The *ACTA1* gene encodes the protein of skeletal muscles, alpha actin 1, which, together with myosin, is responsible for muscle contraction. The *ACTA1* gene in horses consists of 7 exons; exon 1 is noncoding. It spans a region of approximately 2.7 kbp, whereas the transcript length is 1251 bp (ENSEMBL).

Investigations into *ACTA1* gene expression proved that it is one of the most abundant transcripts in the equine skeletal muscle. Higher expressions were observed only in the case of the creatine kinase muscle (*CKM*) and the myosin light polypeptide 1 (*MYL1*) genes. Moreover, the level of *ACTA1* transcripts in Thoroughbred horses trained for 10 months was similar to pretraining levels (5).

In humans, different variants of this gene are associated with congenital myopathies causing muscle weakness (6). Polymorphism in *ACTA1* gene was investigated in cattle in relation to growth traits (7).

This study aimed to detect a polymorphism in introns 5 and 6 of the *ACTA1* gene and to design a polymerase chain reaction (PCR)-based screening test to analyze genetic differentiation depending on breed and utility type.

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2. Material and methods

2.1. Polymorphism detection

The detection of polymorphism was carried out on randomly selected sample of horses belonging to the following breeds: Holstein (n = 4), Polish Cold-Blooded Horse (n = 4), Polish Konik (n = 4), Standardbred (n = 4), Hutsul (n = 4), and Purebred Arabian (n = 4).

Genomic DNA was extracted from blood samples using the MasterPure DNA Purification Kit for Blood Version II (Epicentre). Based on the sequence ENSECAG00000000207 (ENSEMBL) the following primers were designed using Primer3 software: ACTA1F 5'-AGC TGA ACG CGA GAT CGT -3' and ACTA1R 5'-GAG AAT TCG CAC GCT GTT G-3'. PCR reactions were performed in a total volume of 15 µL containing 75–85 ng of DNA, 0.2 mmol of dNTP mix, 1X PCR buffer with NH_4, 12 pmol of each primer, 3.75 mmol $MgCl_2$, and 0.75 U of Taq DNA polymerase (Fermentas). The PCR cycle consisted of 95 °C for 5 min; 32 cycles of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s; and 72 °C for 5 min. Sequencing was performed with both primers separately in an ABI PRISM 3100 Genetic Analyzer. For multiple sequence alignment, ClustalW2 (8) software was used.

2.2. Polymorphism screening

The analysis of genetic differentiation was performed on a sample consisting of 248 horses classified into the following utility types:

- Warm-blooded purebred horses (saddle type): Purebred Arabian (n = 23);
- Warm-blooded half-breed horses (saddle type): Holstein Breed (n = 33), Małopolski Horse (n = 30), Wielkopolski Horse (n = 33);
- Warm-blooded half-breed horses (light-draught type): Standardbred (n = 26);
- Gentle ponies (saddle type): Deutsche Reitpony (n = 20);
- Cold-blooded horses (heavy-draught type): Polish Cold-Blooded Horse (n = 33);
- Primitive horses (general utility type): Polish Konik (n = 33), Hutsul (n = 17).

Samples representing each breed were taken from different stud farms located in Poland. Selected animals were unrelated.

The DNA isolation from blood was carried out using the same method as for the polymorphism detection. Restriction analysis was performed using RestrictionMapper software.

In the case of the PCR-restriction fragment length polymorphism (RFLP) analysis, the same PCR conditions and profile were used as given above. The obtained amplicons (772 bp) were digested with 3 U of the *EcoO109*I enzyme (Fermentas) at 37 °C overnight. Restriction fragments were analyzed using electrophoresis on 2% agarose gels.

For the amplification created restriction site-PCR (ACRS-PCR) analysis, the following primers were designed manually: A1GC (forward) 5'-GCC GAC CGC ATG CAG AAG GA-3' and A1GC (reverse) 5'-TAA GAG AGT GCA GAT AGA GCG TCA CGT-3'. A reverse primer introduced an artificial restriction site through the G to C substitution of the third base at 3'-end. Composition of the PCR ingredients for ACRS-PCR analysis was the same as for PCR-RFLP, with exception of the Taq DNA polymerase amount (1 U). The PCR cycle consisted of 95 °C for 5 min; 30 cycles of 95 °C for 45 s, 64 °C for 45 s, and 72 °C for 1 min; and 72 °C for 5 min. The obtained amplicons (139 bp) were digested with 3 U of the *Eco72*I enzyme (Fermentas) at 37 °C overnight and separated on 3% agarose gels.

2.3. Statistical analysis

For each breed, the following parameters were calculated using PowerMarker v.3.25 (9): frequencies of genotypes and alleles, observed (Ho) and expected (He) heterozygosities, and deviations from Hardy–Weinberg equilibrium (χ^2 test). Haplotypes were estimated using the EM algorithm (10), which estimates the frequency of haplotypes with the maximum likelihood method. For each polymorphism, the polymorphic information content (PIC) was calculated according to Botstein (11).

A nonparametric Kruskal–Wallis test was used to compare genotype frequency between individual breeds (Statistica 9.0 PL). Splice site prediction by neural network was performed using NNSPLICE v.0.9 (12).

3. Results

By sequencing an equine *ACTA1* gene fragment that spans exons 5–7 and introns 5–6 (772 bp), 2 single nucleotide polymorphisms (SNPs) were found: a G > T substitution located in intron 5 (position 1:68411019 relative to ENSEMBL genomic sequence) and a G > C substitution situated in intron 6 (position 1:68411368). Splice site prediction showed that G > T transversion activates a cryptic acceptor splice site.

An analysis of the obtained sequences showed that the *ACTA1* gene is very conservative. The sequenced fragment showed 97% homology to human and dog sequences and 96% homology to pig, cow, and chimpanzee sequences (BLAST/NCBI).

The genotyping of the G > T transversion using the PCR-RFLP method showed the presence of 2 genotypes in the analyzed breeds: *GG* and *GT*. Genotype *GG* was determined based on restriction fragment lengths of 371, 220, and 181 bp, whereas genotype *GT* was defined by lengths of 401, 371, 220, and 181 bp (Figure 1). No *TT* genotype was detected in the investigated breeds. Genotypic frequencies and other population statistics are listed in Table 1.

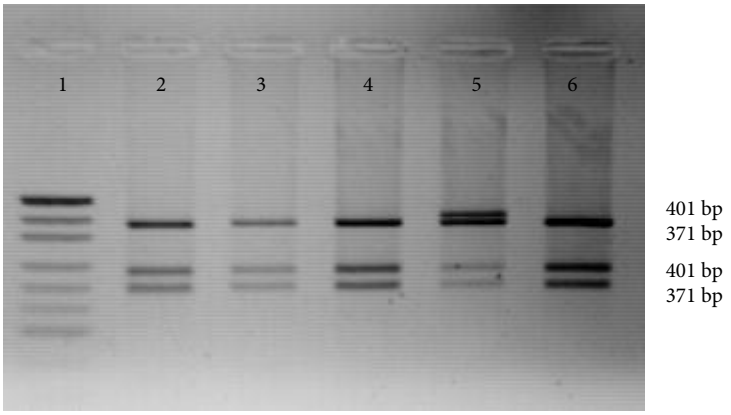

Figure 1. PCR-RFLP analysis of G > T substitution. Lane 1: DNA marker pUC19/*Msp*I (Fermentas); lanes 2, 3, 4, 6: *GG* genotype; lane 5: *GC* genotype.

Table 1. Genotype and allele frequencies with some population genetic indexes calculated for the G > T polymorphism.

Breed	n	Ho	He	χ^2	P	Genotype frequency		Allele frequency	
						GG	*GT*	*G*	*T*
Purebred Arabian	23	0.609	0.423	4.402	0.036	0.391	0.609	0.696	0.304
Holstein Breed	33	0.091	0.087	0.075	0.784	0.909	0.091	0.954	0.046
Małopolski Horse	30	0.233	0.206	0.523	0.469	0.767	0.233	0.883	0.117
Wielkopolski Horse	33	0.212	0.190	0.465	0.496	0.788	0.212	0.894	0.106
Standardbred	26	0.077	0.074	0.042	0.838	0.923	0.077	0.961	0.039
Deutsche Reitpony	20	0.100	0.095	0.055	0.814	0.900	0.100	0.950	0.050
Polish Cold-Blooded Horse	33	0.000	0.000	-	-	1.000	-	1.000	-
Polish Konik	33	0.000	0.000	-	-	1.000	-	1.000	-
Hutsul	17	0.059	0.057	0.016	0.901	0.941	0.059	0.971	0.029

Total: He = 0.145 Ho = 0.135 PIC = 0.126

Frequencies of genotypes were found to be statistically significantly different between the Purebred Arabian and Holstein breeds ($P \leq 0.05$) as well as between the Purebred Arabian, Polish Cold-Blooded Horse, and Polish Konik breeds ($P \leq 0.01$). The distribution of the G > T genotypes was in Hardy–Weinberg equilibrium in all analyzed breeds, but a deviation from the equilibrium ($P \leq 0.05$) was observed in Purebred Arabians. Only Purebred Arabians were characterized by the high heterozygosity. PIC, which is a measure of marker usefulness, was also low.

The ACRS-PCR method applied to genotyping the G > C substitution allowed us to determine 3 genotypes. Genotype *GG* was defined based on the presence of a band of 139 bp and genotype *CC* on the presence of bands of 114 and 25 bp, whereas the heterozygote was characterized by the following band lengths: 139, 114, and 25 bp (Figure 2). Genotype frequencies and other population statistics for the G > C polymorphism are presented in Table 2.

Analysis of genotypic frequencies showed statistically significant differences between some breeds. The largest differences were noted between the Polish Konik and Purebred Arabian, Małopolski Horse, Standardbred, and Holstein breeds ($P \leq 0.01$), as well as between the Polish Konik and Wielkopolski Horse breeds ($P \leq 0.05$). The highest heterozygosity was observed in Deutsche Reitpony, while in Standardbred and Polish Konik it was the lowest. The distribution of the G > C genotypes deviated from the Hardy–Weinberg equilibrium in Małopolski and Purebred

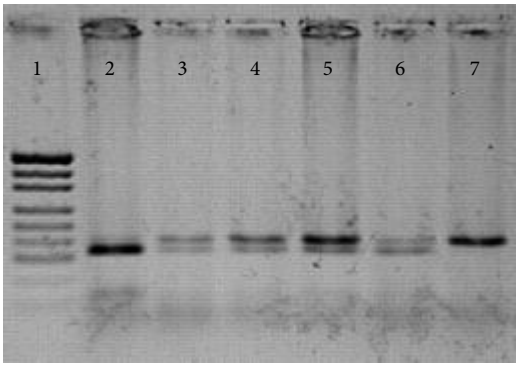

Figure 2. ACRS-PCR analysis of G > C substitution. Lane 1: DNA marker pUC19/*Msp*I (Fermentas); lane 2: *CC* genotype; lanes 3, 4, 5, 6: *GC* genotype; lane 7: *GG* genotype.

Table 2. Genotype and allele frequencies with some population genetic indexes calculated for the G > C polymorphism.

Breed	n	Ho	He	χ^2	P	Genotype frequency			Allele frequency	
						GG	*GC*	*CC*	*G*	*C*
Purebred Arabian	23	0.652	0.439	5.385	0.020	0.348	0.652	-	0.674	0.326
Holstein Breed	33	0.727	0.478	9.029	0.003	0.243	0.727	0.030	0.606	0.394
Małopolski Horse	30	0.633	0.433	6.443	0.011	0.367	0.633	-	0.683	0.317
Wielkopolski Horse	33	0.606	0.496	1.630	0.202	0.242	0.606	0.152	0.545	0.455
Standardbred	26	0.500	0.375	2.889	0.089	0.500	0.500	-	0.750	0.250
Deutsche Reitpony	20	0.950	0.499	16.372	0.000	0.050	0.950	-	0.525	0.475
Polish Cold-Blooded Horse	33	0.606	0.496	1.630	0.202	0.152	0.606	0.242	0.455	0.545
Polish Konik	32	0.500	0.375	3.556	0.059	-	0.500	0.500	0.250	0.750
Hutsul	17	0.588	0.484	0.781	0.377	0.118	0.588	0.294	0.412	0.588

Total: He = 0.496 Ho = 0.632 PIC = 0.373

Arabians ($P \leq 0.05$) and in Holstein and Deutsche Reitpony ($P \leq 0.01$) horses. The PIC value was 0.373 and, according to classification, it was average.

Haplotype analysis (Table 3) showed that the *G-G* and *G-C* haplotypes were common for all analyzed breeds. In primitive horses, the *G-C* haplotype appeared with the highest frequency, whereas the *G-G* haplotype had the lowest one. In the saddle and light-draught types, a reverse relationship was observed. The *T-C* haplotype in cold-blooded and primitive horses was absent or very rare, while in Purebred Arabians it appeared with the highest frequency. The haplotype *T-G* was observed only in the Purebred Arabian and Małopolski Horse breeds.

4. Discussion

The *ACTA1* gene spanning exons 5–7 is the most variable region in domestic animals such as pigs, cows, and dogs (ENSEMBL). In pigs, 4 SNPs were detected in these exons, and 3 SNPs were found in cows and dogs. Interestingly, there are no data on polymorphisms located in introns 5 and 6 in these animals. Graziano et al. (13) analyzed the *ACTA1* gene in patients with nemaline myopathy and found 2 SNPs in intron 2, a repeat element in intron 3, and 3 SNPs in intron 5, which together form an extended haplotype. On the other hand, Laing et al. (6) detected 5 intronic mutations in the *ACTA1* gene in patients; 4 of these mutations affect splice sites and 1 creates cryptic splice site. In our study, we

Table 3. Haplotype estimation based on 2 SNPs.

Breed	Haplotype			
	G-G	*G-C*	*T-C*	*T-G*
Purebred Arabian	0.559	0.137	0.189	0.115
Holstein Breed	0.609	0.344	0.047	-
Małopolski Horse	0.663	0.233	0.077	0.027
Wielkopolski Horse	0.547	0.344	0.109	-
Standardbred	0.750	0.212	0.038	-
Deutsche Reitpony	0.525	0.425	0.050	-
Polish Cold-Blooded Horse	0.469	0.531	-	-
Polish Konik	0.258	0.742	-	-
Hutsul	0.382	0.588	0.030	-

applied a splice site analysis algorithm and found that allele *G* in intron 5 introduces an additional acceptor site, which may affect protein structure and activity.

Hill et al. (14) analyzed some genes selected from a genome scan for Thoroughbred horses. One of these genes was *ACTA1*. These authors' results showed that 2 investigated SNPs located in intron 1 were not associated with the performance phenotype. These SNPs are included in the Equine SNP50 BeadChip (Illumina) and both of them are the T > C substitutions. The first showed higher heterozygosity (0.370) than the second (0.000), as well as a higher frequency of minor alleles (0.145 and 0.007, respectively). The SNPs analyzed in our study showed higher variability and are more informative than those reported by Hill et al. Park et al. (15) investigated another gene, protein kinase adenosine monophosphate activated γ3-regulatory subunit (*PRKAG3*), in horse breeds with widely different phenotypes as regards muscle development and intended performance. One of the discovered variants was only found in horse breeds that can be classified as heavy or moderately heavy, but not in light horse breeds selected for speed or racing performance. On the other hand, Dall'Olio et al. (16) analyzed the horse myostatin gene (*MSTN*) in breeds of different morphological types. They discovered 7 SNPs; 3 of them, which are located in the promoter region, were subjected to further analysis in horses belonging to 16 breeds. The results of their study showed that the g.26C and g.156C alleles appeared with higher frequency in heavy breeds than in light ones. Baron et al. (17) also analyzed the *MSTN* gene in different breeds of horses. Similar to our results, Arabians were shown to have higher haplotype diversity, with one haplotype being specific to this breed. In our study, the haplotype characteristic for Arabians was shared with another breed: the Małopolski Horse, which is known to have Arabian ancestors.

In conclusion, we detected 2 SNPs in the equine *ACTA1* gene and established test procedures based on PCR-RFLP and ACRS-PCR methods in order to screen for polymorphism. Splice site analysis showed that polymorphism G > T is functional. Moreover, we identified some differences, depending on the horse breed and utility type, in the frequencies of genotypes, alleles, and haplotypes.

References

1. Binns MM, Boehler DA, Lambert DH. Identification of the myostatin locus (MSTN) as having a major effect on optimum racing distance in the Thoroughbred horse in the USA. Anim Genet 2010; 41: 154–158.

2. Tozaki T, Miyake T, Kakoi H, Gawahara H, Sugita S, Hasegawa T, Ishida N, Hirota K, Nakano Y. A genome-wide association study for racing performances in Thoroughbreds clarifies a candidate region near the MSTN gene. Anim Genet 2010; 41: 28–35.

3. Schröder W, Klostermann A, Distl O. Candidate genes for physical performance in the horse. Vet J 2010; 190: 39–48.

4. Gu J, Orr N, Park SD, Katz LM, Sulimova G, MacHugh DE, Hill EW. A genome scan for positive selection in thoroughbred horses. PLoS One 2009; 4: e5767.

5. McGivney BA, McGettigan PA, Browne JA, Evans AC, Fonseca RG, Loftus BJ, Lohan A, MacHugh DE, Murphy BA, Katz LM et al. Characterization of the equine skeletal muscle transcriptome identifies novel functional responses to exercise training. BMC Genomics 2010; 11: 398.

6. Laing NG, Dye DE, Wallgren-Pettersson C, Richard G, Monnier N, Lillis S, Winder TL, Lochmüller H, Graziano C, Mitrani-Rosenbaum S et al. Mutations and polymorphisms of the skeletal muscle alpha-actin gene (ACTA1). Hum Mutat 2009; 30: 1267–1277.

7. Tian W, Zhang S, Li X, Gao Q, Jin X, Yan C. Genetic polymorphism and correlation analysis with growth traits of *ACTA1* gene in Yanbian Yellow cattle. Chin Anim Husb Vet Med 2011; 38: 153–157 (article in Chinese with abstract in English).

8. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R et al. ClustalW and ClustalX version 2. Bioinformatics 2007; 23: 2947–2948.

9. Liu K, Muse SV. PowerMarker: an integrated analysis environment for genetic marker analysis. Bioinformatics 2005; 21: 2128–2129.

10. Excoffier L, Slatkin M. Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. Mol Biol Evol 1995; 12: 921–927.

11. Botstein D, White RL, Skolnick M, Davis RW. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 1980; 32: 314–331.

12. Reese MG, Eeckman FH, Kulp D, Haussler D. Improved splice site detection in Genie. J Comp Biol 1997; 4: 311–323.

13. Graziano C, Bertini E, Minetti C, Porfirio B. Alpha-actin gene mutations and polymorphisms in Italian patients with nemaline myopathy. Int J Mol Med 2004; 13: 805–809.

14. Hill EW, Gu J, McGivney BA, MacHugh DE. Targets of selection in the Thoroughbred genome contain exercise-relevant gene SNPs associated with elite racecourse performance. Anim Genet 2010; 41: 56–63.

15. Park HB, Marklund S, Jeon JT, Mickelson JR, Valberg SJ, Sandberg K, Andersson L. Molecular characterization and mutational screening of the PRKAG3 gene in the horse. Cytogenet Genome Res 2003; 102: 211–216.

16. Dall'Olio S, Fontanesi L, Nanni Costa L, Tassinari M, Minieri L, Falaschini A. Analysis of horse myostatin gene and identification of single nucleotide polymorphisms in breeds of different morphological types. J Biomed Biotechnol 2010; 2010: 542945.

17. Baron EE, Lopes MS, Mendonça D, da Câmara Machado A. SNP identification and polymorphism analysis in exon 2 of the horse myostatin gene. Anim Genet 2012; 43: 229–232.