ORIGINAL ARTICLE

ACTN3 genotype distribution across horses representing different utility types and breeds

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Abstract

In horses, the identification of the genetic background of phenotypic variation, especially with regard to performance characteristics and predisposition to effort, has been extensively studied. As α-actinin-3 function is related to the regulation of muscle contraction and cell metabolism, the *ACTN3* gene is considered one of the main genetic factors determining muscle strength. The aim of the present study was to assess the genotype distribution of two SNP variants within the equine *ACTN3* gene (g.1104G > A and c.2334C > T) across different utility types and horse breeds. The analyses were performed on five breeds representing horses of different types, origins and utilities according to performance (in total 877 horses): primitive (Polish koniks; Hucul horses), draught (Polish heavy draught) and light (Thoroughbred and Arabian horses). Two polymorphisms within the *ACTN3* gene locus were genotyped and genotype and allele frequency were compared across populations in order to verify if the identified differences contribute to the phenotypic variation observed in horse breeds. The present study allowed confirmation of the significant differences in genotype distribution of g.1104G > A localized in the promoter region between native breeds and racehorse breeds such as Thoroughbreds and Arabians. The allele/genotype variations between primitive and light breeds confirmed that the analysed variant was under selection pressure and can be correlated with racing ability. Moreover, the significant differences for the c.2334C > T genotype frequency between Arabian horses and other breeds indicate its relationship with endurance and athletic performance. The predominance of the T allele (85%) in Arabians suggests that the T variant was favoured during selection focused on improving stamina and could be one of the genetic factors determining endurance ability. Further research is needed to confirm the association of both polymorphisms with exact racing and/or riding results.

Keywords Actinin-3 · Performance · Polymorphisms · Horse breed · Genetic marker

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Introduction

Alpha-actinins are 100-kDa proteins which belong to the superfamily of cross-linking actin filament proteins. Among them, two muscular-specific isoforms are found, namely, α-actinin-2 and α-actinin-3, as well as 2 non-muscular isoforms, namely, α-actinin-1 and α-actinin-4 [2, 3]. Muscle proteins constitute a part of the contractile mechanism by anchoring actin fibres on the Z line in striated muscle and in dense bodies in smooth muscle cells [13]. In humans, α-actinin-2 occurs in all types of skeletal muscle fibres (I, IIA and IIB), whereas α-actinin-3 is present only in type II fibres, termed fast twitch [12]. The role of α-actinin-3 is crucial during muscle contraction because it is involved in anchoring fibre–actin filaments. In addition, α-actinin-3, due to its spatial position in the cell near the enzyme glycogen

phosphorylase, regulates its activity and influences glycogen metabolism in muscle tissue [1, 16].

In humans, the *ACTN3* gene, which codes for α-actinin-3, is considered the main determining factor for the predisposition to short or long-term effort (sprint or endurance ability). In addition, polymorphisms within the *ACTN3* gene are already used in commercial genetic tests to assess the performance phenotype of athletes and for the optimal selection of training due to individual predispositions [6]. The functional and structural analysis of the *ACTN3* gene is also performed with regard to its association with horse performance characteristics. To date, several reports have focused on the identification of SNP variations in the equine actinin-3 gene in different breeds [10, 24, 26]. Moreover, in horses, the modification of *ACTN3* transcript abundance under training conditions was confirmed [20, 21]. In studies conducted on purebred Arabian horses, a significant decrease in the level of *ACTN3* gene expression in muscle tissue was detected during long-term flat race training using an RNA-seq approach [21] and accurate real-time PCR method [20]. The study of Quinlan et al. 92010) showed that alpha-actinin-3 is related to regulation of glycogen phosphorylase activity resulting in a metabolic shift promoting the increase in fatty acid use in ATP generation. Thus, it can be hypothesized that (decreased of *ACTN3* expression level in muscle can be associated with metabolism regulation [20].

The above reports indicate an important role of α-actinin-3 in the process of adaptation to physical effort. Therefore, there is a need to conduct studies to identify polymorphisms in the *ACTN3* gene in horses, which can be correlated with performance characteristics and could be used in the future as a genetic marker. The aim of present study was to assess the genotype distribution of two SNP variants across different utility types and horse breeds (g.1104G > A and c.2334C > T). The analysed polymorphisms were considered to be potentially related to equine *ACTN3* gene transcription, and the differences in genotype/allele frequency between analysed breeds can contribute to the phenotypic variation observed in horse breeds.

Materials and methods

Animals

A total of 877 horses were included in the study. The analyses were performed on five breeds representing horses of different types, origins and utilities according to performance: primitive, draught and noble light. Samples from primitive included: Polish koniks (n = 182) and Hucul horses (n = 78). Heavy draught included: Polish heavy draught—Polish Coldblood Horse—PKZ (n = 76). Noble light breeds included: Arabian horses (n = 450) and Thoroughbred

horses—TB (n = 91). All samples were previously collected and stored in the Biological Material Bank of the National Research Institute of Animal Production and in the Department of Horse Breeding, Institute of Animal Science, the University of Agriculture in Krakow. The whole blood or hair follicle samples were collected previously as part of other research projects.

Polish koniks

The analysed primitive horses belonging to the Polish koniks breed included representatives of 16 existing maternal and 6 paternal lines. The sampled horses were raised in privet breeders and were included in planned breeding or were representatives of a semi-feral population maintained at the primeval Białowieza Forest. The Polish konik is a native breed presenting behaviour adapted to local environmental conditions, as only these horses are maintained in all possible maintenance systems (herds in conditions close to natural, groups in stable-less systems and individuals in stables). Polish koniks are perfectly adapted to life in forest conditions due to a strong and stocky build, excellent healthiness, and a primitive dun coat and dorsal stripe [23]. They are also used in recreational riding.

Hutsul horses

The second primitive breed, the Hutsul horses, were sampled in State Stud. The first written reference to the supposed Hutsul horses comes from 1603 AD and was published by K. Drohostajski in "Hippika". Hutsul horses were raised in the Eastern Carpathians in the Hutsul region and Bukovina, and in the upper reaches of Czeremosz, Prut, Putilla, Moldova and Suceava. The origin of Hutsul horses it is not clear. It is thought that they are descendants of various types of Tatar horses, Polish, Oriental, Turkish, tarpans, Przewalski horses, and Nrse horses. The breed was consolidated in the 19th century in the Eastern Hutsul region and the Eastern Carpathians under the influence of harsh climate, poor pasture and primitive living conditions. Hutsul is considered one of the oldest primitive breeds maintained in Poland and due to the excellent adaptation to mountain environment conditions are characterized by a strong and lean constitution, longevity, strength and disease resistance [15].

Polish heavy draught

The polish heavy draught horses represent the Sztumski type. Samples were collected from a State stud herd with breeding purposes to provide breeding material to improve locally maintained herds. In fact, the breeding of cold-blooded horses is focused on yielding individuals well suited for draught power and for meat production purposes [18].

The draught horses are heavy boned, with large musculature consisting of short and slow oxidative fibres [5].

Thoroughbred horses

The Thoroughbred horse is one of the most noble breeds in world. Raised more than 300 years ago in UK, the intensive selection focused on a race-athletic phenotype leading to a breed with spectacular achievements and racecourse performance. The samples in this study were collected from horses maintained in state stud with a breeding history since the second world war (IIWW). The individuals are descendants of 36 stallions and 59 dams.

Arabian horses

The analysed Arabian horses represent the population of Polish Arabian horses maintained in State Studs belonging to 16 maternal and 9 paternal lines with a confirmed pure breed of more than 200 years. Arabian horses compete in an equestrian sport, however, they dominate in endurance competitions. Furthermore, they are often used to enrich not only refinement in other horse breeds but also endurance abilities [30]. The selection in Arabians is focused on improving traits related to endurance and athleticism.

Whole blood or hair follicles were collected from the horses. The protocol was approved by the Animal Care and Use Committee of the Institute of Pharmacology, Polish Academy of Sciences in Kraków (no.1173/2015).

SNPs identification

For all horses, two polymorphisms were selected for further evaluation of association with performance characteristics. A previous studies allowed the identification of two Single Nucleotide Polymorphisms (SNPs)—one in the promoter region (g.1104G > A; rs1144978872) [24] and one in the 19th exon located near a splice region in the junction between the 18th and 19th exons (c.2334C > T) (c.2334C > T; rs1141508235) [20], which were detected in Arabian horse population. Both SNPs can potentially affect the transcription process of the *ACTN3* gene.

The polymorphisms were genotyped using the PCR–RFLP method according to the procedure presented in Table 1. The PCR product was obtained using Phanta Master Mix and High-Fidelity PCR (Vazyme Biotech Co., Ltd) according to the manufacturer's protocol with an annealing temperature at 57 °C. After amplicon digestion, the PCR products were separated on 3% and 4% agarose gels (for g.1104G > A and c.2334C > T; respectively).

The Hardy–Weinberg equilibrium was calculated using the Court Laboratory HW calculator (Court's 2005–2008 online calculator; [4]). The differences in genotype distribution between breeds were estimated using the Chi square test according to (Preacher [14]).

Table 1 The detail inforamtion about analyzed polymorphisms

Gene region	Chromosome location	Accession rs# number/HGVS	Identyfication method	Alleles	N	Primers
Promoter region	26511704	g.1104G > A rs1144978872	HgaI	G 528 A 453, 75	720	F CTAGAG CCCCTC CCACTC TC R GGC TGCATA ACCATC ATCATC
19 exon	26523860	c.2334C > T rs1141508235	HpyCH4III	C 141, 110 T 251	877	F AGG AGCAGC TCAACG AGTTC R ATGTAG GAGGGA GGCACT GA

SNPs nomenclature was shown according to HGVS (Human Genome Variation Society) and based on NW_001867370.1 reference (for SNP in promoter region) and ENSECAG00000018961 (for the 19th exon SNP)

N number of analyzed samples

Results and discussion

Genotype and allele distribution of g.1104G > A SNP

The genotyping of the g.1104G > A polymorphism in a total of 877 horses showed significant differences in genotype and allele frequencies across the analysed breeds. The three native and coldblooded breeds were characterized by the predominance of the AA genotype and the low number of GG horses (Table 2; Fig. 1). The lowest number included animals with the GG genotype in both native Polish koniks and Hucul horses (1% and 1.9% respectively). The opposite genotype distribution was detected in noble breeds: Arabian and Thoroughbred had GG horses accounting for 47.7% in both analysed populations (Fig. 1). In TB horses, no AA horses were identified. Furthermore, both Arabian and Thoroughbred populations were not consistent with HWE equilibrium (Table 2). The analysis of allele and genotype distribution confirmed the significant (p < 0.0001) differences between light breeds and other breeds. Moreover, both light breeds also differed between each other (genotype distribution—p < 0.035; alleles frequency p < 0.0001) (Fig. 2; Table 3).

Genotype and allele distribution of c.2334C > T SNP

The analysis of the c.2334C > T allele and genotype distribution showed less variation between the investigated breeds than the g.1104G > A polymorphism. A similar genotype frequency was identified for Polish koniks and Thoroughbreds. For Hutsul horses, three genotypes were also identified; the most abundant were heterozygotes. In the Polish coldblood population, only CC and TC horses were identified (Fig. 3; Table 2). For most breeds, a higher frequency was detected for the CC genotype (form 86.6% for PKZ to 42.6% for Hutsul horse). The opposite genotype frequency was observed in the Arabian horse population, where CC horses accounted for 2% of all horses (Fig. 3). This was confirmed by differences in C–T allele distribution across analysed breeds and the Arabian horse population (<0.0001) (Fig. 4; Table 4).

Discussion

The association between muscle histological structure (the percentage and diameter of different fibre types) and predisposition to a specific type of effort (sprint or endurance) has been established [27, 31]. The percentage of individual muscle fibres and their diameter depend on many factors, including environmental factors (nutrition or breeding) as well as genetic background [28]. Studies conducted in

Table 2 The genotypes frequency and number of horses in individual group of both investigated SNPs

Genotypes	Polish konik			Hucul horses			PKZ			Thoroughbred			Arabian horses		
	AA	AG	GG	AA	AG	GG	AA	AG	GG	AA	AG	GG	AA	AG	GG
g.1104G > A rs1144978872 (promotor region)															
Number of horses	64	39	1	37	16	1	14	14	5	–	46	42	60	234	147
Percentage of genotypes	61.5%	37.5%	1.0%	68.5%	29.6%	1.9%	42.4%	42.4%	15.2%	–	52.3%	47.7%	13.6%	53.1%	33.4%
HWE significance	p value	0.0595		p value	0.6242		p value	0.6321		p value	0.0009		p value	0.0286	
Minor allell frequency	G		0.2	G		0.17	G		0.36	G		0.74	G		0.6
	CC	TC	TT	CC	TC	TT	CC	TC	TT	CC	TC	TT	CC	TC	TT
c.2334C > T rs1141508235 (19th exon)															
Number of horses	155	22	5	36	39	3	66	10	–	67	22	2	9	117	324
Percentage of genotypes	85.2%	12.1%	2.7%	46.2%	50.0%	3.8%	86.8%	13.2%	–	73.6%	24.2%	2.2%	2.0%	26.0%	72.0%
HWE significance	p value	0.0009		p value	0.0541		p value	0.5392		p value	0.9026		p value	0.6774	
Minor allell frequency	T		0.09	T		0.29	T		0.07	T		0.14	T		0.85

Fig. 1 The genotype distribution of g.1104G > A polymorphism across five analyzed horse breeds (PKZ—Polish heavy draught)
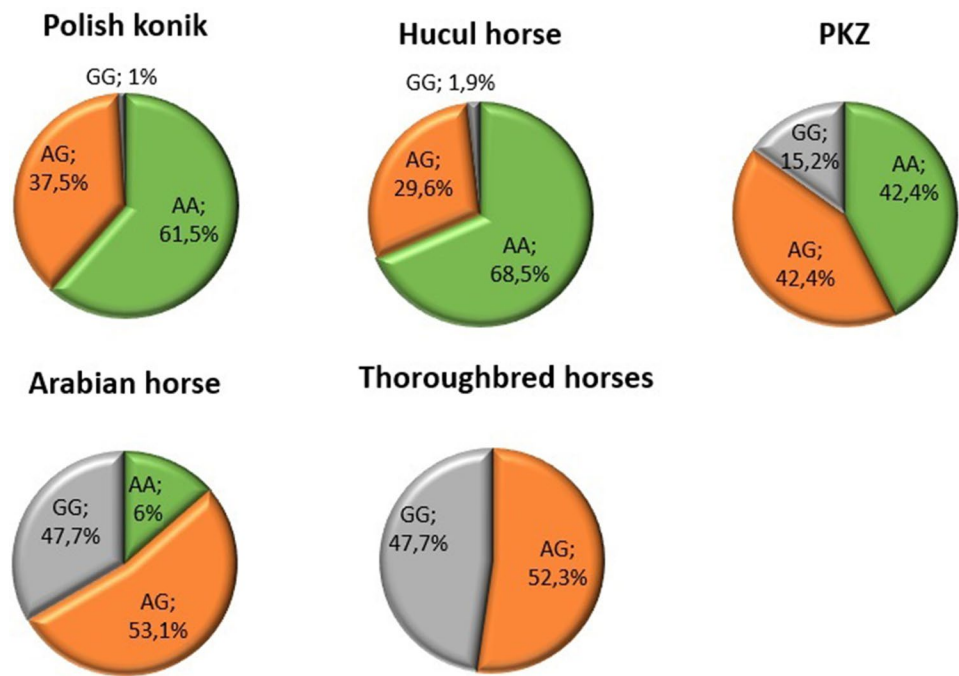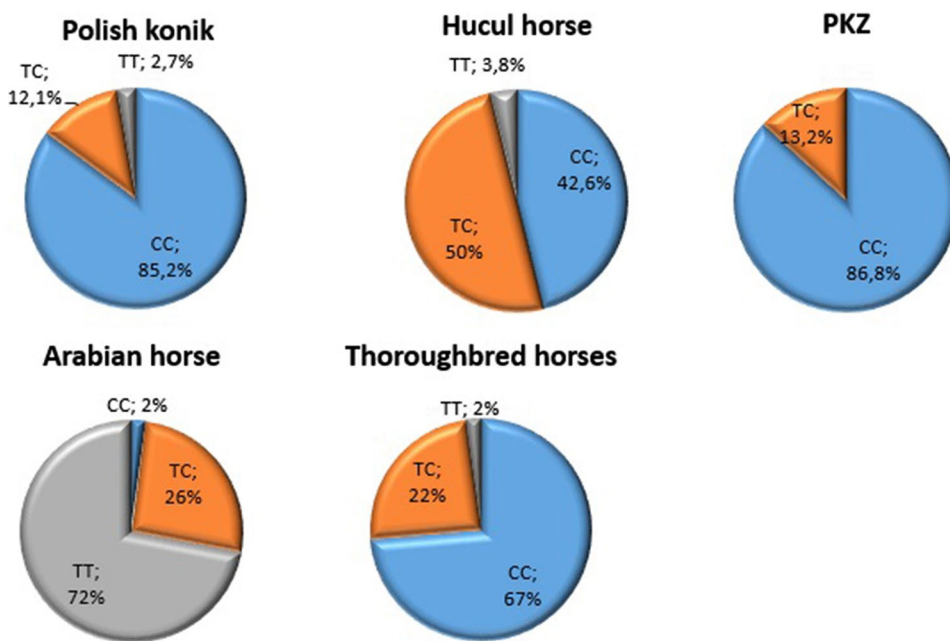

Fig. 2 The alleles distribution of 5′UTR variant - g.1104G > A across horse breeds (PKZ—Polish heavy draught)
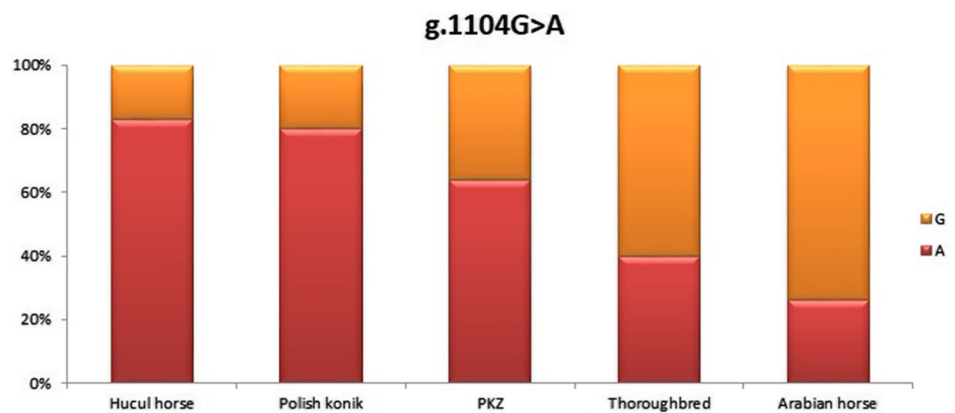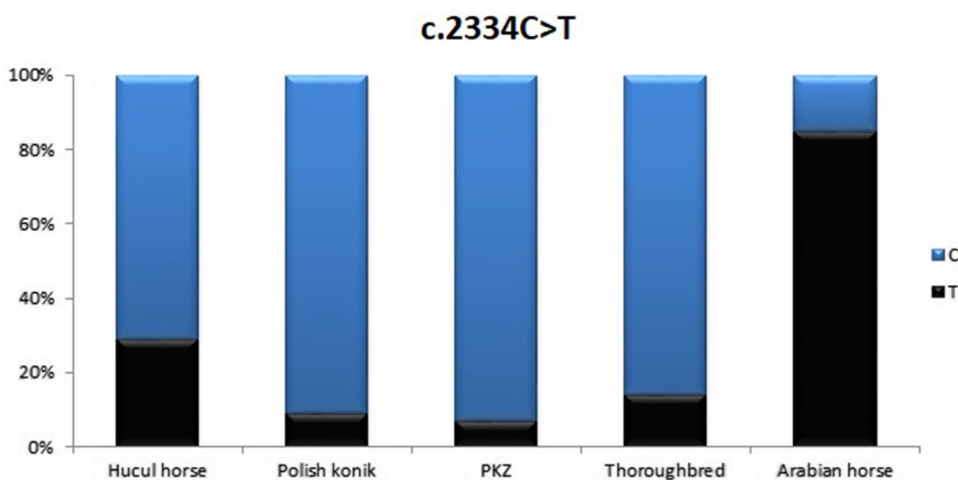

Table 3 Differences in genotypes and alleles distribution between analyzed breeds in the term of g.1104G > A polymorphism

	Polish konik	Hucul	PKZ	Arabian	Thoroughbred
Allele distribiution					
Polish konik	*	ns	0.117	< 0.0001	< 0.0001
Hucul	ns	*	0.002	< 0.0001	< 0.0001
PKZ	0.117	0.002	*	< 0.0001	< 0.0001
Arabian	< 0.0001	< 0.0001	< 0.0001	*	0.035
Thoroughbred	< 0.0001	< 0.0001	< 0.0001	0.035	*
Genotypes distribution					
Polish konik	*	ns	0.0002	< 0.0001	< 0.0001
Hucul	ns	*	0.006	< 0.0001	< 0.0001
PKZ	0.0002	0.006	*	< 0.0001	< 0.0001
Arabian	< 0.0001	< 0.0001	< 0.0001	*	< 0.0001
Thoroughbred	< 0.0001	< 0.0001	< 0.0001	< 0.0001	*

Fig. 3 The genotype distribution of c.2334C > T polymorphism across five analyzed horse breeds (PKZ—Polish heavy draught)

Fig. 4 The alleles distribution of c.2334C > T localized in 19th exon across horse breeds (PKZ—Polish heavy draught)

Table 4 Differences in genotypes and alleles distribution between analyzed breeds in the term of c.2334C > T polymorphism

	Polish konik	Hucul	PKZ	Arabian	Thoroughblood
Allele distribiution					
Polish konik	*	0.003	ns	< 0.0001	ns
Hucul	0.003	*	ns	< 0.0001	ns
PKZ	ns	ns	*	< 0.0001	ns
Arabian	< 0.0001	< 0.0001	< 0.0001	*	< 0.0001
Thoroughblood	ns	ns	ns	< 0.0001	*
Genotypes distribution					
Polish konik	*	ns	ns	< 0.0001	ns
Hucul	ns	*	0.03	< 0.0001	ns
PKZ	ns	0.03	*	< 0.0001	ns
Arabian	< 0.0001	< 0.0001	< 0.0001	*	< 0.0001
Thoroughblood	ns	ns	ns	< 0.0001	*

ns not significant

humans have shown the relationship between the proportion of specific types of muscle fibres and the type of physical exercise performed. In people practicing strength discipline or runners, fast-twitch fibres predominate, while in endurance sports, slow-twitch fibres are more abundant [11, 27, 31]. The α-actinin-3 is fibre type specific and presents only in type II fast twitch fibres [12]. Moreover, a significant correlation was found between the gene encoding α-actinin-3 with the percentage of particular types of muscle fibres [25]. Due to α-actinin-3 localization and function, the fast fibre-specific gene *ACTN3* has been proposed as one of the critical genetic factors determining skeletal muscle function and metabolism [1].

In humans, the role of the *ACTN3* gene has been thoroughly studied, making it possible to determine its effect on the phenotype associated with physical exercise. The mutation present in the *ACTN3* gene, located in position R577X (rs1815739), has a significant impact on the predisposition of the unit for short and fast or long-distance runs. This mutation occurs in a large part of the population, in which it causes a deficiency of the α-actinin-3 protein. It has been established that the R577X polymorphism directly leads to a change in the features of muscle fibres. The R577X mutation is located at position 577 of the gene encoding α-actinin-3 and results in the conversion of cytosine to thymine, C > T. As a consequence, the amino acids sense change and instead of the appearance of the next amino acid–arginine, a premature STOP codon is created. Studies conducted in humans have shown that α-actinin-3 is absent in 18% of healthy, light-skinned individuals due to the presence of the homozygous TT genotype [29]. The association of *ACTN3* R5777X polymorphism with power and sprint performance has been established by numerous researches [6, 8, 9, 17]. On the other hand, the relation of R5777X to endurance performance [17, 19, 22] and its genetic model of inheritance (dominant, additive, recessive) [7] still have not been completely known.

The huge potential of using the *ACTN3* gene as a genetic marker in athletes led to its analysis in other species, especially in horses, which are evolutionarily adapted to effort and are a perfect model for adapting the body to exercise and training. The sequence of the horse *ACTN3* gene encoding alpha-actinin-3 was previously studied by Mata et al. [10] and Thomas et al. [24]. Based on sequencing of the *ACTN3* gene performed in 15 horses, Thomas et al. [24] identified 34 SNPs. Most of them (28 SNPs) were located in the non-coding region, and the remaining 6 polymorphisms in exons were discovered as silent changes. The authors also identified several new DNA sequence variations, of which, g.1104G > A was proposed as the most interesting, with a potentially large effect on the function of the alpha-actinin-3 protein due to its location in the 5′UTR region and with a different frequency of this change in the breeds of horses

with different predispositions to effort. Thomas et al. [24] hypothesized that the 5′UTR variant polymorphism may affect horse racing characteristics by modifying the level of *ACTN3* gene expression. This hypothesis was supported by Ropka-Molik et al. [20], which showed that flat-racing training in Arabian horses significantly modified *ACTN3* gene expression regardless of the analysed splice isoforms. Moreover, the authors indicated a possible effect of the g.1104G > A polymorphism on *ACTN3* gene expression in the *gluteus medius* muscle in horses after an intense gallop phase [20].

In 2014, Thomas et al. presented the genotype and allele frequency of the *ACTN3* g.1104G > A SNP in sprint (Thoroughbred), endurance (Arabian), pace (Starnardbred) and strength (Clydesdale, Shire) types horses. The research confirmed the differences in both genotype and allele distributions between analysed breeds. The warmblood horses such as Thoroughbred and Arabian horses have a higher percentage of the G allele - 83% and 62%, respectively, while Standardbred and Clydesdale/Shire horses (coldblooded breeds) have only 31% and 23% of the G allele, respectively [24]. The results obtained in the present study confirmed the low frequency of the G allele in the native breed (Polish koniks and Hutsul) and Polish heavy draught (20%, 17% and 36%, respectively), 60% in Thoroughbreds and 74% in Arabian horses. Similar to the report by Thomas et al. [24], only the GG and AG genotypes were identified in TB horses. The opposite genotype frequency was detected for the muscular and powerful horse breeds Clydesdale and Shire, where no GG horses were found and the majority of the analysed group were AA animals (86%). Interestingly, in the present study the predominance of AA horses was identified in both native breeds, Polish konik (65%) and Hucul horses (69%), while in the Polish heavy draught population AA accounted for only 42%. The analysis of all five horse populations showed a significant increase in the G allele frequency from the native breed throughout coldblood to sprint and endurance types (from 17% in Hucul to 74% in Arabian). Such an identified trend indicated an association of allele A with the primitive type of horse breed and strength. On the other hand, the significant predominance of the G allele in warmbloods, especially Arabian horses, directed it to racehorse breeding.

With regard to the second analysed SNP c.2334C > T localized in the 19th exon, highly significant differences were observed between Arabian horses and other breeds (p < 0.0001). In Arabian horses, the frequency of the T allele was 85%, while in sprint type TB horses it was only 14% and only 7% in Polish heavy draught horses. In the coldblood population, no TT horses were identified. Surprisingly, no significant differences in allele and genotype distributions were obtained between Thoroughbred and other breeds, where the CC horses predominated. The c.2334C > T

polymorphism is a synonymous variant with a potential low effect on protein structure or gene function. However, the analysed SNP is localized in the vicinity of the splice region at the exon junction and can be related to modification of precursor messenger RNA into mature messenger RNA in the splicing process. Arabian horses are characterized by unusual strength, stamina and dominance in endurance riding. The selection in this breed is focused on improving traits related to endurance and athleticism. Therefore, the distribution of the c.2334C > T genotype strongly indicates that the T allele can be essential for endurance ability, while the lack of a difference between sprint type horses and native and coldblooded breeds showed that the investigated SNP is not associated with sprint performance traits.

Both analysed polymorphisms (g.1104G > A; c.2334C > T) were also identified in Chinese native Yili Horses [26]. In this analysed breed, the authors showed a similar genotype distribution of the g.1104G > A polymorphism as found in our Arabian horse population (for Yili horses AG–44.74 and GG–42.11% and for Arabian horses AG–53.1, GG–47.7%). Furthermore, for c.2334C > T SNP genotype frequency (CC–86.84%; CT–10.53%; TT–2.63%), the Yili Horse population was the most similar to Polish koniks and completely opposite of Arabian horses. Wang et al. [26] focused on another SNP missense variant, g.9059T > G, identified in this breed and proposed it as a clausal mutation affecting athletic performance (TT–94.74% and TG–5.26%). The authors showed that g.9059T > G changed the secondary structure of α-actinin-3, and proposed this SNP as a candidate for breeding improvement towards racing traits.

Conclusion

The present study allowed a comparison of the allele and genotype frequencies of *ACTN3* polymorphisms across horses representing different utility types and breeds. The results confirmed the significant differences in genotype distribution of g.1104G > A localized in the promoter region between native breeds and racehorse breeds such as TB and Arabians. Such discrepancies indicated that g.1104G > A was under selection pressure since horse domestication is focused on improving desired features. The allele/genotype variations between primitive and light breeds show that the analysed variant is related to racing ability. Furthermore, highly significant differences in the genotype frequency of c.2334C > T between Arabian horses and other breeds can indicate its relationship with endurance and athletic performance. The predominance of the T allele (85%) in Arabians could suggest that the T variant was favoured during selection focused on improving stamina and could be one of the genetic factors determining endurance ability. Further

research is needed to confirm the association of both polymorphisms with exact racing and/or riding results.

Compliance with ethical standards

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