A single nucleotide polymorphism in exon 3 of the *myostatin* gene in different breeds of domestic pigeon (*Columba livia* var. *domestica*)

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ABSTRACT: Myostatin is considered to be one of the most powerful negative regulators of muscle growth. The lack of a functional myostatin (MSTN) or its mutation can result in uncommon musculature like "double-muscling" in Belgian Blue cattle. Recent studies on *MSTN* variability indicate its relationship with racing performance in dogs and racehorses. Considering the high homology of *MSTN* among the vertebrates, there are grounds to suppose that the same correlation will occur in pigeons. The aim of this study was to analyse *MSTN* variability in several pigeon breeds raised for different purposes. The PCR-RFLP method was used for genotyping the C → T silent substitution in exon 3 of the *MSTN* gene. A total of 376 domestic pigeons (*Columba livia* var. *domestica*) were genotyped. The differences in genotype frequencies ($P < 0.01$) and allele frequencies ($P < 0.01$), between the studied groups were observed. Minor allele ($MSTN^T$) frequency was the highest in the group of utility pigeons (0.291), which are characterised by abundant muscle mass and higher body mass-to-muscle mass ratio. Further studies should be performed in order to determine the impact of the SNP analysed in the present paper on the amount of functional myostatin in muscles.

Keywords: myostatin; polymorphism; synonymous mutation; muscle growth

Pigeons are now bred mainly for recreational purposes and have no real economic significance. Features such as spatial orientation, muscle strength, stress resistance and endurance of flight, are essential characteristics of homing (sport) pigeons (Jerolmack 2007). Overall physical capacity plays a crucial role in long-distance flights. It is defined as the ability to undergo long-lasting physical exertion without any signs of tiredness followed by rapid recovery of expended energy resources. Muscle tissue seems to be a fundamental factor which determines endurance parameters (Cassano et al. 2009).

Muscle mass is strictly regulated by various factors and can adapt to physical exertion by increasing the amount and size of contractile proteins (Leiter et al. 2011). One of the most powerful negative regulators of muscle growth is myostatin (McPherron and Lee 1997). Inhibition of myostatin signalling in skeletal muscle resulted in increased lean mass, decreased fat mass and improved glucose metabolism (Guo et al. 2009).

Although little is known about the genetic determinants that underlie athletic performance, two attributes, strength and speed, almost certainly have a major role in determining athletic success (Lee 2007). Mosher et al. (2007) examined the role of *MSTN* in determining the physical characteristics and athletic capabilities of dogs and found that loss of MSTN function enables these whippets to run faster. Previous studies with mice have also demonstrated that an increase in muscles correlates with increased strength (Whittemore et al. 2003). However, the dog study was the first to demonstrate conclusively that the increased muscling resulting from the absence of MSTN can translate into enhanced athletic performance (Lee 2007). In horses, Hill et al. (2010a, b) described an association between optimum racing distance and an SNP in the equine *MSTN* gene, provided evidence that the g.66493737C>T polymorphism in equine *MSTN* is the most powerful genome-wide predictor of optimum racing distance in Thoroughbred Flat racehorses.

Recent studies on polymorphisms of candidate genes in pigeons create the possibility for its use in marker-assisted selection (Dybus et al. 2006, 2008; Dybus and Haase 2011). Research on the mechanisms of action of myostatin has indicated that myostatin critically influences the formation of endurance traits, especially, in case of its inactivation or decreased expression level. The main goal of this study was to analyse *MSTN* gene polymorphism as a potential marker for endurance traits in homing pigeons.

MATERIAL AND METHODS

A group of 376 domestic pigeons: 144 racing pigeons from Natural Antwerp Breeding Station (Belgium), 117 specimens of flying/fancy pigeons from local breeders and 115 utility pigeons (37 from local breeders and 78 individuals of Wroclaw Meat from Department of Genetics of Wrocław University of Environmental and Life Sciences). The details concerning breeds used in the study are presented in Table 1.

Genomic DNA was isolated from blood samples (5 µl) using the MasterPure™ DNA Purification Kit (Epicentre Biotechnologies). The SNP in the domestic pigeon *MSTN* gene was previously detected by DNA sequencing (performed at the Institute of Biochemistry and Biophysics, PAS, Warsaw, Poland) and deposited in GenBank database (HM749880).

Genotypes of pigeons were determined using the PCR-RFLP method. In the first step of this study, PCR primers were designed to produce a 185 base pairs amplification product using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi):
MSTN3e-F 5’-GCAGAGATTTTGGCCTTGAC-3’
MSTN3e-R 5’-GAGGTGAGTGTGCGGGTATT-3’

The PCR mixture contained ~40 ng of DNA template, 10 pmol of each primer, 1 × PCR buffer, 1.5mM $MgCl_2$, 200µM dNTP and 0.3 units of recombinant *Taq*-polymerase in a total volume of 12 µl. The following temperature cycles were applied: denaturation at 94°C/5 min, followed by 35 cycles at 94°C/30 s, primer annealing at 61°C/40 s, amplicon synthesis at 72 °C/30 s and final synthesis at 72 °C/5 min. After amplification, specificity and efficiency of the PCR reaction (4 µl) was evaluated by electrophoresis of the products in 1.5% agarose gels (PRONA) in 1 × TBE and digestion (8 µl) with 2 units of *Btg*I re-

Table 1. Characteristics of the studied populations

Homing (*n* = 144)	Flying and fancy (*n* = 117)		Utility (*n* = 115)
	Flying (*n* = 72)	Fancy (*n* = 45)	
Janssen (*n* = 48)	German Long Faced Tumbler (*n* = 16)	Fantail (*n* = 22)	Wroclaw Meat (*n* = 78)
Thone (*n* = 24)	German Nun (*n* = 10)	Capuchin (*n* = 8)	Strasser (*n* = 15)
van Dyck (*n* = 12)	Danzig Highflier (*n* = 8)	German Show Homer (*n* = 6)	King (*n* = 14)
de Smet-Matthys (*n* = 12)	Vienna Kiebitz (*n* = 6)	Cauchois (*n* = 5)	Hungarian (*n* = 4)
Stichelbaut (*n* = 12)	Carrier (*n* = 6)	Maltese (*n* = 4)	Polish Lynx (*n* = 4)
Grondelaers (*n* = 12)	Polish Barb (*n* = 6)		
Wanroy (*n* = 12)	German Magpie (*n* = 5)		
Meulemans (*n* = 6)	Polish Owl (*n* = 4)		
Bricoux (*n* = 6)	Polish Helmet (*n* = 4)		
	Bagdad of Nuremberg (*n* = 4)		
	Polish Short-Beaked (*n* = 3)		

striction endonuclease (NEB). Restriction fragments were separated through 3% agarose gels (PRONA). DNA in gels was stained with Ethidium bromide.

RESULTS

The following DNA fragments were observed due to the *MSTN/Btg*I polymorphism: 185 bp for the *MSTN/Btg*I^{TT} genotype (no digestion), 118 and 67 bp for the *MSTN/Btg*I^{CC} and 185, 118 and 67 bp for the heterozygotic genotype (Figure 1). The molecular basis of the analysed SNP is a C → T transition, located in the 3rd exon of the *MSTN* gene in the 287th codon (ACC to ACT) for threonine. It is a silent mutation (Figure 2).

The frequency of *MSTN*^{T} in all studied pigeons was low (0.149), but in the group of utility pigeons the rare gene variant was more frequent (0.291) than in the two other group ($P = 0.0000$). The detailed data regarding allele/genotype frequencies in the studied group of pigeons is presented in Table 2. Statistically significant differences in genotypes ($\chi^2 = 45.25$; $P < 0.01$) and allele frequencies ($\chi^2 = 22.53$; $P < 0.01$), between the studied groups were observed (Table 3).

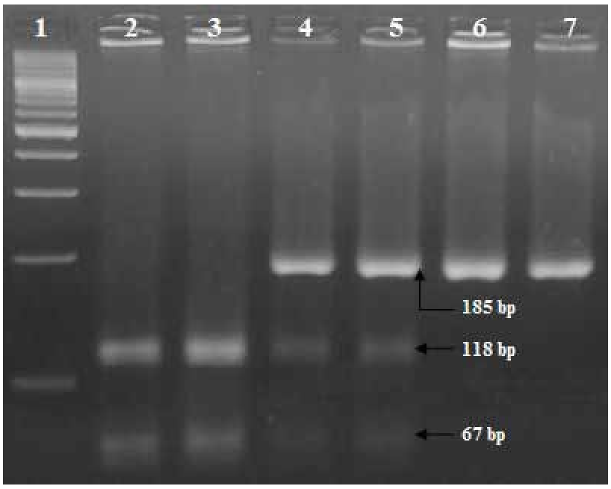

Figure 1. Electrophoretic analysis after *Btg*I digestion

Lane 1 = DNA marker (GeneRuler™ 100 bp Plus, MBI Fermentas), lanes 2, 3 = *MSTN/Btg*I^{CC}, lanes 4, 5 = *MSTN/Btg*I^{CT}, lanes 6, 7 = *MSTN/Btg*I^{TT}

The distribution of genotypes in the groups of homing and flying/fancy pigeons were similar, with a very low frequency of the rare *MSTN*^{T}, except for the Vienna Kiebitz (0.333) breed. The highest frequency of *MSTN*^{T} was detected in Strasser – 0.464, but more than 50% of animals were heterozygotic. A similar trend was observed in the most numerous group of utility pigeons, Wroclaw Meat (47.4% were heterozygotic). More information regarding allele and

Table 2. Frequencies of genotypes/alleles in studied pigeons

Group	*n*	Genotype			Allele	
		MSTN^{TT}	*MSTN*^{CT}	*MSTN*^{CC}	*MSTN*^{T}	*MSTN*^{C}
Homing	144	0.007 ($n = 1$)	0.174 ($n = 25$)	0.819 ($n = 118$)	0.094	0.906
Flying and fancy	117	0.025 ($n = 3$)	0.103 ($n = 12$)	0.872 ($n = 102$)	0.077	0.923
Flying	72	0.042 ($n = 3$)	0.069 ($n = 5$)	0.889 ($n = 64$)	0.076	0.924
Fancy	45	–	0.156 ($n = 7$)	0.844 ($n = 38$)	0.078	0.922
Utility	115	0.078 ($n = 9$)	0.426 ($n = 49$)	0.496 ($n = 57$)	0.291	0.709
Total	376	0.035 ($n = 13$)	0.229 ($n = 86$)	0.736 ($n = 277$)	0.149	0.851

Table 3. The results of the chi-square test

Group comparison	Genotypes		Alleles	
	χ^2	*P*	χ^2	*P*
Homing – flying and fancy	2.75	n.s.	5.17	*
Homing – utility	31.3	**	10,32	**
Flying and fancy – utility	40.39	**	26.88	**

*$P < 0.05$, **$P < 0.01$, n.s. = not significant

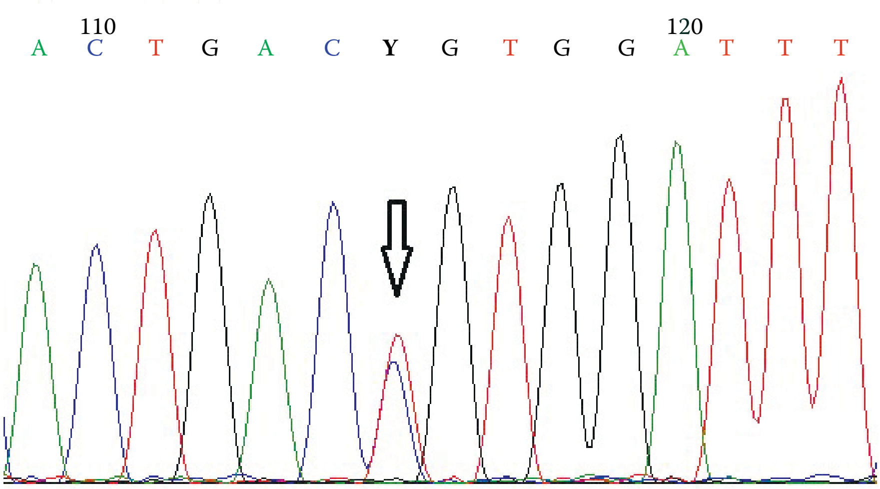

Figure 2. The results of DNA sequencing (heterozygotic genotype). The arrow indicates the SNP located in the 287th codon in the 3rd exon of the pigeon *MSTN* gene

genotype frequencies for particular breeds included in the groups of homing, flying, fancy and utility pigeons is given in Tables 4, 5, 6 and 7, respectively.

DISCUSSION

In line with our expectations, the observed genotype distribution and allele frequencies among the analysed groups of pigeons were significantly different. The rare allele ($MSTN^T$) was much more frequent within the group of utility pigeons, in comparison with the other groups. It must be noted that individuals derived from utility breeds are characterised by abundant muscle mass and higher body mass-to-muscle mass ratio. On the basis of these facts, the hypothesis can be formulated that the SNP discussed in the present study may influence myostatin-related negative regulation of muscle growth.

Myostatin gene polymorphism and its potential influence on various traits have been investigated in many species. In two Norwegian sheep breeds, two distinct *MSTN* gene mutations are linked to carcass conformation and obesity. SNPs were identified in noncoding regulatory regions of the ovine and porcine *MSTN* gene. Some of them influence *MSTN* gene expression levels, which can be correlated with growth, muscle mass and carcass performance traits (Dall'Olio et al. 2010). Zhang et al. (2011) discovered four substitutions (G2283A, C7552T, C7638T and T7661A) in the *MSTN* gene of the poultry breed, Bian. They reported that *EE*

Table 4. Frequencies of genotypes/alleles in the group of homing pigeons

Line	Genotype			Allele	
	$MSTN^{TT}$	$MSTN^{CT}$	$MSTN^{CC}$	$MSTN^{T}$	$MSTN^{C}$
Janssen	– –	0.167 ($n = 8$)	0.833 ($n = 40$)	0.083	0.917
Thone	0.042 ($n = 1$)	0.083 ($n = 2$)	0.875 ($n = 21$)	0.083	0.917
Van Dyck	– –	0.167 ($n = 2$)	0.833 ($n = 10$)	0.083	0.917
De Smet-Matthys	– –	0.167 ($n = 2$)	0.833 ($n = 10$)	0.083	0.917
Stichelbaut	– –	0.167 ($n = 2$)	0.833 ($n = 10$)	0.083	0.917
Grondelaers	– –	0.333 ($n = 4$)	0.667 ($n = 8$)	0.167	0.833
Wanroy	– –	0.250 ($n = 3$)	0.750 ($n = 9$)	0.125	0.875
Bricoux	– –	0.167 ($n = 1$)	0.833 ($n = 5$)	0.083	0.917
Meulemans	– –	0.167 ($n = 1$)	0.833 ($n = 5$)	0.083	0.917

Table 5. Frequencies of genotypes/alleles in the subgroup of flying pigeons

Breed	Genotype			Allele	
	$MSTN^{TT}$	$MSTN^{CT}$	$MSTN^{CC}$	$MSTN^{T}$	$MSTN^{C}$
German Long Faced Tumbler	– –	0.125 (*n* = 2)	0.875 (*n* = 14)	0.062	0.938
German Nun	– –	– –	1.000 (*n* = 10)	–	1.000
Danzing Highflier	– –	0.125 (*n* = 1)	0.875 (*n* = 7)	0.063	0.937
Vienna Kiebitz	0.333 (*n* = 2)	– –	0.667 (*n* = 4)	0.333	0.667
Carrier	0.167 (*n* = 1)	– –	0.833 (*n* = 5)	0.167	0.833
Polish Barb	– –	– –	1.000 (*n* = 6)	–	1.000
German Magpie	– –	0.200 (*n* = 1)	0.800 *n* = 4)	0.100	0.900
Polish Owl	– –	– –	1.000 (*n* = 4)	–	1.000
Polish Helmet	– –	– –	1.000 (*n* = 4)	–	1.000
Bagdad of Nuremberg	– –	0.250 (*n* = 1)	0.750 (*n* = 3)	0.125	0.875
Polish Short-Beaked	– –	– –	1.000 (*n* = 3)	–	1.000

Table 6. Frequencies of genotypes/alleles in the subgroup of fancy pigeons

Breed	Genotype			Allele	
	$MSTN^{TT}$	$MSTN^{CT}$	$MSTN^{CC}$	$MSTN^{T}$	$MSTN^{C}$
Fantail	– –	0.136 (*n* = 3)	0.864 (*n* = 19)	0.068	0.932
Capuchin	– –	0.250 (*n* = 2)	0.750 (*n* = 6)	0.125	0.875
German Show Homer	– –	0.333 (*n* = 2)	0.667 (*n* = 4)	0.167	0.833
Cauchois	– –	– –	1.000 (*n* = 4)	–	1.000
Maltese	– –	– –	1.000 (*n* = 4)	–	1.000

Table 7. Frequencies of genotypes/alleles in the group of utility pigeons

Breed	Genotype			Allele	
	$MSTN^{TT}$	$MSTN^{CT}$	$MSTN^{CC}$	$MSTN^{T}$	$MSTN^{C}$
Wroclaw Meat	0.064 (*n* = 5)	0.474 (*n* = 37)	0.462 (*n* = 36)	0.301	0.699
Strasser	0.200 (*n* = 3)	0.533 (*n* = 8)	0.267 (*n* = 4)	0.464	0.536
King	– –	0.143 (*n* = 2)	0.857 (*n* = 12)	0.071	0.929
Hungarian	– –	0.500 (*n* = 2)	0.500 (*n* = 2)	0.250	0.750
Polish Lynx	0.250 (*n* = 1)	– –	0.750 (*n* = 3)	0.250	0.750

and *DE* genotypes are characterised by higher body weight than the *DD* genotype in individuals between six and 18 weeks of age. The G2283A mutation located in exon I was considered as potential genetic marker for body weight. Mutations in the myostatin coding sequence have been described to result in body weight gain, but have also been linked to increased mortality rates. On the basis of this fact, it can be concluded that the *MSTN* gene probably has a pleiotropic character. All of the above mentioned mutations can be regarded as very valuable genetic markers, which can improve incomes from animal breeding in the future (Ye et al. 2007).

The variability in the myostatin coding sequence affects not only traits such as body weight gain and higher muscle mass-to-fat mass ratio. According to Mosher et al. (2007) it can also be associated with increased physical capacity. These researchers have revealed that deletion of two nucleotides in the whippet *MSTN* gene results in premature occurrence of a stop codon and the synthesis of an incomplete myostatin, which consists of 313 amino acids and which is 17% smaller in size in comparison to the native MSTN form. This leads to changes in muscle tissue structure, which affects sprinting capacity in these dogs. Individuals with at least one mutated allele were characterised by better musculature and enhanced speed in performed experiments. Hill et al. (2010b) obtained very interesting results, while studying the *GDF-8* gene in the Thoroughbred horse breed. They observed differences in nucleotide sequences, based on a C → T substitution, within groups of horses racing at different distances. Individuals with a *CC* genotype were characterised by an unusually high speed and reached the best results over short distance races. Heterozygotic animals (*CT*) showed the best performances in medium distance races, while *TT* individuals showed unusual endurance. The SNP in the equine *MSTN* gene described by Hill et al. (2010a, 2010b) may be used to predict the genetic potential of a horse, although the SNP is not a classical (termination codon type) functional variant. The authors hypothesises that the described mutation or its haplotypic background has an impact on *MSTN* gene expression.

Although, this C to T substitution analysed in this study is located in an exonic region of the *MSTN* gene, it does not result in an amino acid change, because both ACC and ACT encode threonine. This type of mutation is called synonymous and according to the dogma, it is assumed to be functionally neutral. However, recent studies on this matter have revealed that synonymous SNPs can probably widely influence processes linked to gene expression and protein folding (Sauna et al. 2007). Sorensen and Pedersen (1991) have indicated that the GAA codon is translated 3.4 fold faster than the GAG codon in *Escherichia coli*, although both codons encode alanine. The amount of cognate tRNAs that surround ribosomes during translation is proportional to the frequency of codon usage, so availability of tRNAs for infrequent codons is decreased in comparison to frequent ones. Therefore, synonymous SNPs can influence the overall rate of translation and its kinetics. Abnormal translation kinetics, arising from a single nucleotide polymorphism, may lead to the production of a protein with a different final structure and function (Komar 2007). Kimchi-Sarfaty et al. (2007) have reported that a synonymous mutation in the *Multidrug Resistance* 1 (*MDR*1) gene results in a conformational change in the P-glycoprotein (P-gp), encoded by this gene. As a result, P-gp shows different substrate specificity. Pagani et al. (2005) have indicated that synonymous SNPs affect the splicing of the *CFTR* gene and the skipping of exon 12. Thus, it seems that synonymous mutations can have much more significance than was previously thought and should be not omitted in genetic linkage studies.

The main prizes in homing pigeon competitions now reach up to 200 000 US dollars. Depending on race distance, the pigeon breeders choose individuals characterised by certain appropriate features. Developing our understanding of the regulation of *MSTN* gene expression and the mechanism of action of myostatin can potentially open up many exciting avenues. In part, it can help in the raising of new pigeon varieties or in the improvement of existing breeds, helping them to take top positions in pigeon racing competitions (Jerolmack 2007).

The increasing financial value of pigeon breeding worldwide is a strong reason for scientists to give more attention to this species. The present study is the first, in which the structure of the gene encoding myostatin in domestic pigeons was analysed. Polymorphism in the *MSTN* gene seems to be a very promising candidate in the search of a genetic marker for determining predispositions to racing in pigeons. In order to determine the impact of the SNP analysed in this paper on the amount of functional myostatin in muscles, further studies should be performed.

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