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Research Article

Mitochondrial DNA D-loop sequence variation among 5 maternal lines of the Zemaitukai horse breed

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Abstract

Genetic variation in Zemaitukai horses was investigated using mitochondrial DNA (mtDNA) sequencing. The study was performed on 421 bp of the mitochondrial DNA control region, which is known to be more variable than other sections of the mitochondrial genome. Samples from each of the remaining maternal family lines of Zemaitukai horses and three random samples for other Lithuanian (Lithuanian Heavy Draught, Zemaitukai large type) and ten European horse breeds were sequenced. Five distinct haplotypes were obtained for the five Zemaitukai maternal families supporting the pedigree data. The minimal difference between two different sequence haplotypes was 6 and the maximal 11 nucleotides in Zemaitukai horse breed. A total of 20 nucleotide differences compared to the reference sequence were found in Lithuanian horse breeds. Genetic cluster analysis did not shown any clear pattern of relationship among breeds of different type.

Key words: D-loop, *Equus caballus*, phylogeny, polymorphism.

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Introduction

Until recently conservation efforts have focused on wild species, but now domesticated animals are recognized as an important part of biodiversity and more efforts to save rare breeds are made. The Zemaitukai horse (*Equus caballus*) is a native breed of Lithuania. As a consequence of unfavourable historical and economic circumstancies, the number of Zemaitukai seriously declined and by 2003 the total population size was 147 animals. According to pedigree data, the Zemaitukai horse population now consists of two stallion (male) and five mare (female) family lines (Macijauskiene, 2002). Out of the five mare families the one of Kastanke is represented by significant number of individuals. The least numerous is the Mirta family. The other three are the Zibute, Arabe and Tulpe maternal families. Genetic relationship between Lithuanian and other horse breeds is a point of interest, as the origin of the breed is not exactly known.

Mitochodrial DNA (mtDNA) has strictly maternal inheritance (Hutchinson *et al.*, 1974), which means mtDNA haplotypes should be shared by all individuals within a maternal family line. Mitochondrial DNA is useful for studying the evolution of closely related species and many studies have focused on the mitochondrial D-loop region, the most variable part of mtDNA (Ishida *et al.*, 1994) due to a higher substitution rate than in the rest of the mtDNA genome (Cann *et al.*, 1984). The entire horse mtDNA sequence has been reported (Xu & Arnason, 1994). Stability of maternal inheritance within documented horse pedigrees has been demonstrated in Lipizzan (Kavar *et al.*, 1999) and Arabian horses (Bowling et al, 2000). Mitochondrial DNA sequence polymorphism has been used to examine genetic relationship within breeds (Hill *et al.*, 2002; Luis et al 2002), among breeds (Kim *et al.*, 1999; Mirol *et al.*, 2002), between domestic and wild horse populations (Oakenfull & Ryder, 1998) and also to address questions of horse domestication (Lister *et al.*, 1998; Vila *et al.*, 2001).

Here we present a study designed to examine the validity of the breed pedigree, measure mtDNA diversity, and examine interbreed relationships, as this information could be useful in conservation and management of this rare horse breed.

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Materials and Methods

Samples from Zemaitukai (ZO) mare families were: Arabes - Austeja ZRg59, Mirtos - Asveja Zrg173, Kastankes family - Kanarele ZRg40, Zibutes - Zemyna ZRg172 and Tulpes - Tola ZRg217, respectively. Three random samples from other two Lithuanian horse breeds Zemaitukai Heavy Type (ZH) and Lithuanian Heavy Draught (LH)

were sequenced. In order to determine phylogenetic relationship three random samples from Finn horse (FH), Gotland (GT), Hanovarian (HA), Holstein (HO), Hucul (HU), Polish Heavy (PH), Wielkopolski (PI), Polish Primative (PO), Posavina (PV) and Trakehner (TK) breeds were sequenced. These are breeds that were available to us from the same general geographical area as Lithuania. DNA was extracted from fresh blood samples or frozen serum samples using the Puregene DNA extraction kit (Gentra Systems) following the manufacturer instructions. Primers from published horse mtDNA sequence (Xu and Arnason, 1994) were designed: Forward 5'-CGCACA TT ACCCTGGTCTTG-3', Reverse 5'-GAACCAGATGCCA GGTATAG-3'.

Polymerase chain reaction (PCR) was carried out in 25 μL total reaction volumes, each containing 0.2 mM dNTP's, 0.5 μM of each primer, 2.5 mM $MgCl_2$, 1XPCR buffer, 1 U of Taq polymerase (PE Applied Biosystems, MA), 1 U of ApliTaq Gold (PE Applied Biosystems, MA) and 50 ng of template DNA. The reaction mixture was heated to 95 °C for 5 min, followed by 30 cycles each consisting of 40 s denaturation at 94 °C, 45 s annealing at 55 °C, 45 s of extension at 72 °C and then a final 10 min extension at 72 °C.

Sequencing was carried out using BigDye™ Terminator Cycle Sequencing Kit (PE Applied Biosystems, MA). Sequences were determined using the ABI Prism 377 DNA Sequencer. All sequences were confirmed by re-sequencing the same sample using a second independent PCR reaction. Sequence alignment was performed using reference equine mtDNA sequence (GeneBank X79547). Genetic clustering and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar *et al.* 2001). In addition to the breeds listed above sequences belonging to other horse breeds from GeneBank (http://www.ncbi.nlm.nih.gov/GenBank) were incorporated into the analysis. The GeneBank accession numbers for selected sequencies are AY246174-AY246200, AY246209-AY246252 and AY246259-AY246271. The sequence of *Equus prewalski* was used as outgroup.

Results and Discussion

Table 1 shows polymorphic sites in the control region between the five maternal lineages of Zemaitukai horse breed, Lithuanian Heavy Draught, Zemaitukai Heavy Type and the reference sample - GeneBank X79547 (Xu and Arnason, 1994). Sequence analysis of 421bp revealed five different haplotypes in the Zemaitukai horse breed supporting the presumed maternal pedigree lineages for existing horses. A total of 20 nucleotide differences compared to the reference sequence were found in Lithuanian horse breed. All detected mutations were transitions. The minimal difference to the unrelated horse reference sequence was six nucleotides in the Tulpes and Zibutes families and the maximal difference was nine in Arabes family for Zemaitukai horse breed. The minimal difference between two different haplotype DNA sequences was 6 and the maximum was 11 nucleotides. Out of five samples analyzed five different haplotypes were found for Zemaitukai horses, which is comparable to other findings [13 haplotypes in 16 maternal lines of Lippizzan horses (Kavar *et al.*, 1999), 27 haplotypes in 34 Arabian maternal lines (Bowling *et al.*, 2000)].

For other breeds, out of three randomly selected samples, two haplotypes for Finn horse, Holstein, Hucul, and

Table 1 - Polymorphic sites of Zemaitukai horse breed family lines and reference (GeneBank X79547) in control region of horse mtDNA D-loop sequence. See text for abbreviations.

	15494	15495	15496	15534	15542	15585	15597	15602	15603	15604	15617	15635	15639	15649	15650	15659	15666	15667	15703	15720	15726	15740	15771	15777	15806	15807	15809	15810	15811
X79547	**T**	**T**	**A**	**C**	**C**	**G**	**A**	**C**	**T**	**G**	**T**	**C**	**T**	**A**	**A**	**T**	**G**	**A**	**T**	**G**	**G**	**A**	**C**	**A**	**C**	**C**	**A**	**A**	**C**
ZO																													
Arabes		C						T											C	A	A	G	T	G					T
Mirtos		C						T			C					C				A			T		T				
Kastankes		C			T		G	T				T					A		C	A									
Zibutes		C				A									G		A			A								G	
Tulpes		C					G	T					C		G					A									
ZH																													
ZH1		C					G	T		A								G	C	A			T	G		T	G		
ZH2		C			T		G	T				T					A		C	A									
LH																													
LH1	C	C	G	T		A			C					G						A			T						
LH2		C					G	T							G					A									
LH3		C			T		G	T				T			G		A		C	A									

Zemaitukai Heavy type and three haplotypes for rest of the breeds were observed. A total of 38 nucleotide differences compared to the reference sequence were found representing 9% of the total DNA sequence analyzed. Out of 38 detected mutations, there were 37 transitions and a single deletion in one individual of the Polish primitive horse breed. The deletion was located in stretch of five cytosines at position 15532.

Table 2 shows nucleotide differences between reference sample and horse breeds tested, Kimura's two-parameter genetic distances showed high variation within and between the breeds. Nucleotide diversity ranged from 0.0129 in the Gotland to 0.0244 in Trakehner and Hanovarian horse breeds. Nucleotide diversity of the Zemaitukai was 0.0199. Low nucleotide diversity in Gotland breed is consistent with the low autosomal genetic diversity found in this breed. High nucleotide diversity within Lithuanian horse breeds is consistent with the results obtained by blood typing and DNA typing (Juras *et al.*, 2003). The Zemaitukai horse has high nucleotide and sequence diversity despite having experience recent bottleneck. The intense interbreeding, due to migrations during formation of different horse breeds, or persistence of several mitochondrial archetypes within the breeds might have caused the high intrabreed nucleotide diversity in the horse (Kavar *et al.*, 1999).

For genetic cluster analysis, sequences of Arabian, Thoroughbred, Friesian, Akhal-Teke, Belgian, Caspian, Cleveland Bay, Clydasdales, Exmoor, Garrano, Haflinger, Lusitano, Noriker, Shetlands and Sorraia horse breeds were obtained from gene bank and included with the breeds sequenced in this study for analysis. The breeds represent a wide geographic area as well as the different horse types. Sequences were truncated to 353 bp for this analysis in order to maximize the number of breeds included. Both maximum parsimony and neighbour-joining analysis showed similar patterns. After truncating sequences for genetic cluster analysis, breeds that shared the same haplotype were pooled in to groups named C1, C2, C3 and C4. The C1 group contained the Holstein, Trakehner, Lithuanian Heavy Drought, Arabian, Sorraia and Hannovarian breeds. Hanovarian, Trakehner, Zemaitukai and Arabians were put in C2. Haplotypes of Akhal-Teke, Belgian, Haflinger, Zemaitukai, Polish Heavy, Garrano, Cleveland Bay, and Noriker were placed in C3. Hucul, Thoroughbred, Haflinger, Noriker and Lithuanian Heavy drought horse haplotypes formed the C4 group. Genetic cluster analysis did not shown any clear pattern of relationship among the domestic horse breeds whose relationships are well known historically (bootstrap analysis of 1000 replications, Figure 1). Haplotypes from the same breed frequently clustered in separate groups that included breeds of completely different horse breed types. This same pattern has been seen in other studies of horse mtDNA (Vila *et al.*, 2001; Jansen *et al.*, 2002), but are different from ones obtained using blood and protein typing data, were horse breeds clustered corresponding to their known ancestry (Juras *et al.*, 2003). The lack of a pattern may be attributed the high rate of evolution in the control region, which may cause a site to mutate once, and then mutate later changing back to the original haplotype. The result would be the loss of an informative site. Also migration of individuals from one population to another before the establishment of the stud books or at the initial stage of breed formation. The mtDNA analysis provided no new information about the ancestry of the Lithuanian horse breeds.

The analysis of mitochondrial DNA of Zemaitukai horses adds useful information for the effective management and conservation of this rare breed. The mtDNA re-

Table 2 - Distances calculated by Kimura two-parameter method. Distances within the groups are given in bold. Nucleotide differences between reference and horse groups are given in italic. See text for abbreviations.

	X79547	FH	GT	HA	HO	HU	LH	PH	PI	PO	PV	TK	ZH	ZO
X79547		*14*	*10*	*17*	*10*	*11*	*16*	16	*13*	*15*	*16*	*17*	*14*	*20*
FH	0.0256	**0.0170**												
GT	0.0153	0.0248	**0.0129**											
HA	0.0194	0.0282	0.0159	**0.0244**										
HO	0.0170	0.0269	0.0137	0.0154	**0.0145**									
HU	0.0158	0.0269	0.0149	0.0166	0.0195	**0.0219**								
LH	0.0186	0.0265	0.0162	0.0165	0.0154	0.0159	**0.0228**							
PH	0.0203	0.0248	0.0167	0.0214	0.0253	0.0158	0.0206	**0.0194**						
PI	0.0107	0.0248	0.0145	0.0198	0.0228	0.0141	0.0189	0.014	**0.0162**					
PO	0.0203	0.0191	0.0162	0.0230	0.0236	0.0199	0.0217	0.0178	0.0168	**0.0178**				
PV	0.0219	0.0265	0.0167	0.0220	0.0269	0.0158	0.0211	0.0151	0.0146	0.0179	**0.0178**			
TK	0.0203	0.029	0.0156	0.0166	0.0162	0.0166	0.0168	0.0211	0.0200	0.0228	0.0212	**0.0244**		
ZH	0.0232	0.0170	0.0215	0.0224	0.0183	0.0257	0.0208	0.0257	0.0248	0.0183	0.0273	0.0233	**0.0219**	
ZO	0.0175	0.0227	0.0153	0.0188	0.0160	0.0192	0.0183	0.0206	0.0190	0.0193	0.0226	0.0193	0.0197	**0.0199**

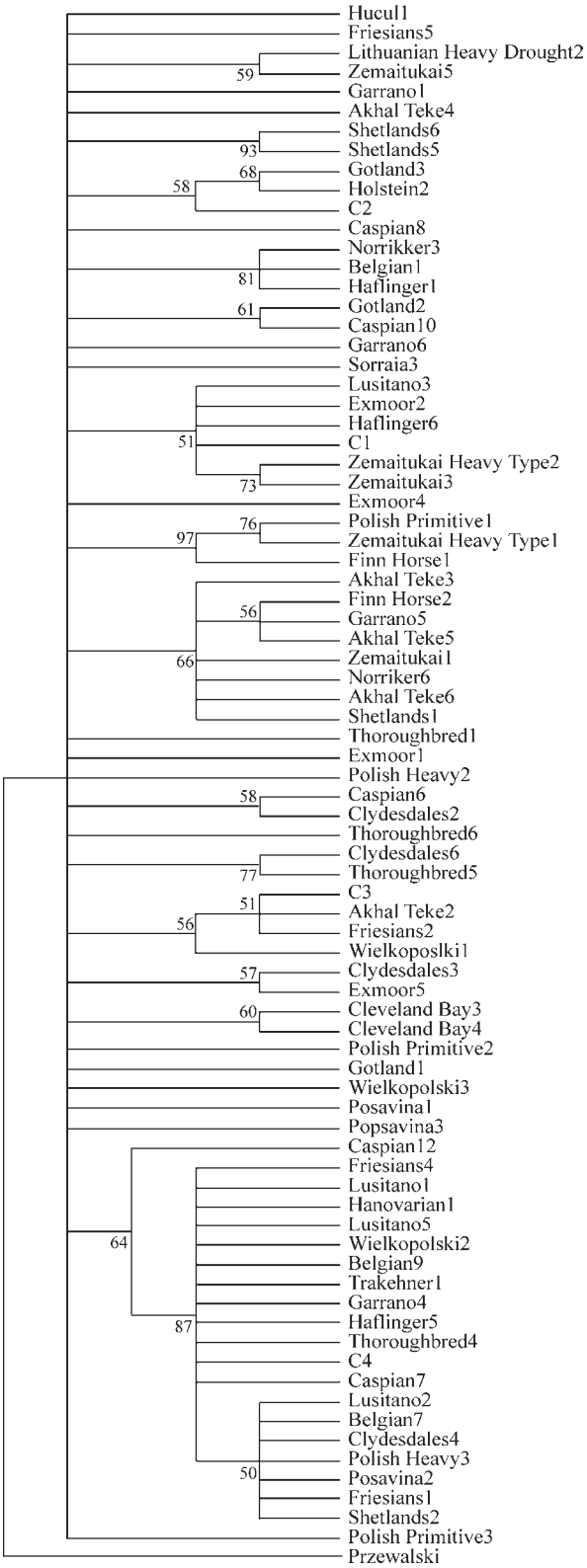

Figure 1 - Neighbour-joining tree with 1000 bootstrap replication for Lithuanian and other European horse breeds.

sults confirm at least five remaining maternal lineages in Zemaitukai horse breed, which are useful for development of breeding strategies, aimed at evening the genetic contribution of different maternal founding lineages. The mtDNA data also provides additional insights into the genetic diversity of the breed, which, in combination with data from nuclear genes, can be used to maximize the maintenance of genetic diversity within the Zemaitukai horse.

Accession numbers

GenBank accession numbers AY575103-AY575139 for sequences obtained in this study are as follows: FH1-2, GT1-3, HA1-3, HO1-2, HU1-2, LH1-3, PH1-3, PI1-3, PO1-3, PV1-3, TK1-3, ZH1-2 and Z01-5.

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