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Full Length Research Paper

A study of patrilineal genetic diversity in Iranian indigenous horse breeds

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Autosomal markers and mtDNA have been used in horse phylogenetic studies. These studies display evolutionary events that happened in both sexes or only in females. It is necessary to investigate genetic diversity in Y-specific markers for clarifying contribution of males in horse domestication. The Y chromosome, excluding the pseudoautosomal region, is inherited as a single nonrecombinant unit and, therefore, it warrants that mutational events in patrilines are preserved as single haplotypes. Six Y-specific microsatellites were used to study patrilineal genetic variation in 405 male horses from 8 Iranian native horse breeds, one wild population and an exotic breed. These markers displayed no variation in all populations. The lack of polymorphisms could be as a result of lower contribution of stallions to the gene pools of the domestic horses compared to the mares because a sex bias is towards females due to a special breeding strategy (in which a few selected stallions mate with many mares each), a strong tendency to upgrade many breeds by crossing between champion stallions from particular breeds and mares from different breeds; also due to a bias in the early utilization of male horses as food source, a bias towards stallions in migrations and lack of detailed maps on horse Y chromosome.

Key words: Iranian horse breeds, microsatellite, patriline, polymorphism, Y chromosome.

INTRODUCTION

In horses, autosomal markers and mtDNA have been used in phylogenetic relationship and domestication studies. Analysis of horse mtDNA has displayed a high level of genetic variation between maternal lineages (Jansen et al., 2006; Vila et al., 2001; Oakenfull et al., 2000). It implies that a large number of wild mares participated as founders in domestic horse gene pool. Also, the genetic variations and relationships between different horse populations have been studied with autosomal microsatellites (Luis et al., 2007; Glowatzki-Mullis et al., 2005; Achmann et al., 2004; Aurich et al., 2003; Cuningham et al., 2001). The results from autosomal markers and mtDNA studies display evolutionary events that happen in both sexes or only in females. But, it is necessary to investigate genetic diversity in Y-Specific markers to clarify contributions of males in horse domestication, as those of females were considered by mtDNA (Santani, 2004).

The horse Y chromosome euchromatic region approximately contains 15 Mb (of the total 45-50 Mb) and is placed in the distal one-third of the long arm. The pseudoautosomal region (PAR) lies at the end of this arm. Other regions of this chromosome are heterochromatic (Raudsepp et al., 2004). The Y chromosome is male specific and effectively haploid. The entire segments of this chromosome, excluding the pseudoautosomal region, are inherited as a single

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nonrecombinant unit. Hence, it warrants that mutational events in patrilines are preserved as single haplotypes.

Despite the existence of extensive sequence data on human and mouse Y chromosome, very low information is available in this field for other mammals (Hurles and Jobling, 2001). Most efforts to identify variations in domestic horse breeds at geographically wide range were unsuccessful (Santani, 2004; Wallner et al., 2003, 2004; Lindgren et al., 2004). Only in a study on Chinese domestic horse breeds, polymorphism was reported in one horse Y-specific microsatellite (Ling et al., 2010).

Iran has a long history in horse domestication and breeding (Andrews and Legates, 1962; Tavakkolian, 1999). Iranian horse breeds may be classified into 4 main groups according to their origins and habitats: North alluvial plains such as Caspian breed, northeast fields such as Turkmen breed, west highlands such as Kurd breed and southwest and central plateau such as Persian Arab breed (Tavakkolian, 1999).

We used six Y-specific microsatellite markers (Wallner et al., 2004) to study patrilineal genetic diversity in 8 Iranian native horse breeds, one wild horse population of Iran and an exotic horse breed.

MATERIALS AND METHODS

Samples and DNA extraction

Blood samples from 405 male horses were collected from 8 Iranian native domestic horse breeds {Caspian (n = 49), Qashqai (Dareh-Shoori) (n = 25), Guilani (n = 34), Kurd (n = 55), Mazandarani (n = 51), Persian Arab (Asil) (n = 98), Qarehbagh (n = 7), and Turkmen (n = 69)}, one wild horse population in north of Iran (n = 3) and an exotic breed (Thoroughbred (n = 14)), in 19 provinces of Iran (Table 1). Geographical distribution of these samples is displayed in Figure 1. Also, 10 female samples were used as negative control (one sample per population). Total genomic DNA was extracted from whole blood using AccuPrep® Genomic DNA Extraction Kit (Bioneer, Republic of Korea).

Microsatellite loci and PCR

Six Equus Y-specific microsatellite markers (Wallner et al., 2004) were used to study genetic variation in Iranian horse populations. Forward primers were labeled at 5'-end with fluorescent dyes (Table 2).

PCR amplifications were performed in 20 µl reactions each containing 40 ng genomic DNA, 2 mM $MgCl_2$ (Fermentas, Canada), 250 µM of each dNTP (Roche Applied Science, Germany), 0.03 µM of both forward and reverse primers (Metabion, Germany), 1X PCR buffer (Fermentas, Canada) and 0.5 U Taq DNA polymerase (Fermentas, Canada). Amplifications were performed using the GeneAmp PCR 9700 (Applied Biosystems, USA). PCR conditions were as follows: an initial denaturation for 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 40 s at 58 or 62°C (Table 2), 90 s at 72°C and a final extension of 30 min at 72°C.

Genotyping

Genotyping was performed using Applied Biosystems 3130 Genetic Analyzer, based on capillary electrophoresis. Briefly, 1 µl of each PCR product was mixed with 0.3 µl GeneScan™-500 LIZ Size Standard (Applied Biosystems, USA) as an internal standard and 10 µl Hi-Di™ Formamide (Applied Biosystems, USA) as a diluter. After denaturation for 3 min at 95°C, samples were run with 3130 Genetic Analyzer based on Fragment Analysis 36_POP7_1 run module and G5 dye set. The GeneMapper v.4.0 software (Applied Biosystems, USA) was used for allele size determination. PCR amplification and genotyping were repeated once, when any samples failed.

RESULTS AND DISCUSSION

All extracted genomic DNA displayed a sharp band without contamination on agarose gel. The quantity of samples was controlled by nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Each sample was diluted to 20 ng per µl based on nanodrop spectrophotometer provided concentrations. PCR amplifications were optimized to both material concentrations and cycling conditions. Both DNA amount for PCR amplification and PCR product concentration used for capillary electrophoresis were optimized to reach optimal peak sizes and shapes. Electrophorograms displayed the typical peak pattern of dinucleotide microsatellites in which a main peak was preceded by one or two stutter peaks (Wallner et al., 2004). The allele fluorescence intensities were fallen between 1000 to 4000 RFU.

There was no variation in six horse Y-specific microsatellites on all Iranian horse populations. The male samples displayed a single allele, whereas none was seen in the female samples (Table 3).

In the case of horses, three studies have tried to identify polymorphisms on Y chromosome, but no polymorphic microsatellites (Wallner et al. 2004) and SNPs (Lindgren et al., 2004; Wallner et al. 2003) were found in horse Y chromosome. In another research, one polymorphic microsatellite was identified on horse Y chromosome (MS30); however, this marker was not Y-specific and amplified in both male and female samples (Santani, 2004). Finally, in a recent study, Ling et al. (2010) used six horse Y-specific microsatellites isolated by Wallner et al. (2004) to study genetic variation in Chinese horse breeds. The five markers displayed no polymorphisms in these horses. Although two haplotypes were detected in one Y-specific microsatellite (Eca.YA16). This was the first paper that reported variation in horse Y-specific markers.

Lack of polymorphisms on horse Y-specific microsatellites is clearly in contrast to the genetic variation frequently observed on the autosomal microsatellites (Luis et al., 2007; Glowatzki-Mullis et al. 2005; Achmann et al., 2004; Aurich et al., 2003; Cuningham et al., 2001) and mitochondrial DNA (Jansen et al., 2006; Vila et al., 2001; Yang et al., 2002; McGahern, et al., 2006; Kakoi et al., 2007). In addition, our results did not confirm the polymorphism that has been observed on the human Y chromosome (Kayser et al., 2004) and some of mammalians such as bovine(Edwards et al., 2000) and ovine (Meadows et al., 2004). These horse Y-specific microsatellites showed polymorphism in other Equidae;

Table 1. Horse breeds, their abbreviations and sample size with separation based on provinces and regions of Iran.

Breed	Abbreviation	Province (region) of Iran	Sample size
Caspian	CA	Tehran (Middle)	19
		Guilan (North)	9
		Alborz (Middle)	21
Qashqai (Dareh-Shoori)	DS	Isfahan (Middle)	10
		Fars (South)	3
		Kerman (Southeast)	12
Guilani	GU	Guilan (North)	34
Kurd	KU	Kermanshah (West)	20
		Kurdistan (West)	9
		Isfahan (Middle)	19
		West Azerbaijan (North West)	3
		East Azerbaijan (North West)	4
Mazandarani	MZ	Mazandaran (North)	51
Persian Arab (Asil)	PA	Khuzestan (Southwest)	27
		Yazd (Middle)	23
		Kermanshah (West)	8
		Alborz (Middle)	14
		Hamadan (West)	5
		Kerman (Southeast)	1
		East Azerbaijan (North West)	20
Qarehbagh	QB	East Azerbaijan (North West)	4
		West Azerbaijan (North West)	2
		Ardabil (North West)	1
Turkmen	TU	North Khorasan (North East)	37
		Golestan (North)	18
		Khorasan-e-Razavi (East)	14
Thoroughbred [a]	TB	Alborz (Middle)	5
		Golestan (North)	5
		Khorasan-e-Razavi (East)	4
Guilan wild horse	GW	Guilan (North)	3

[a] Introduced from other countries; such as UK, Australia, Turkey, etc.

as a result, the single haplotypes observed for them could not be resulted from a low mutation rate alone in horse Y chromosome (Wallner et al., 2004).

The presence of a single Y chromosome haplotype in the horse may reflect a significantly lower contribution of stallions to the gene pool of domestic horses than the mares (Santani, 2004; Wallner et al., 2003). The effective population size of the Y chromosome depends on the number of stallions in a population. On the other hand, the Y chromosome is hemizygous and found only in males. Consequently, under random mating conditions, the effective population size of the Y chromosome is 1/3 compared to X chromosome and 1/4 compared to autosomes. The sex bias towards females further reduces the effective population size of the horse Y chromosome. Hence, a limited number of this

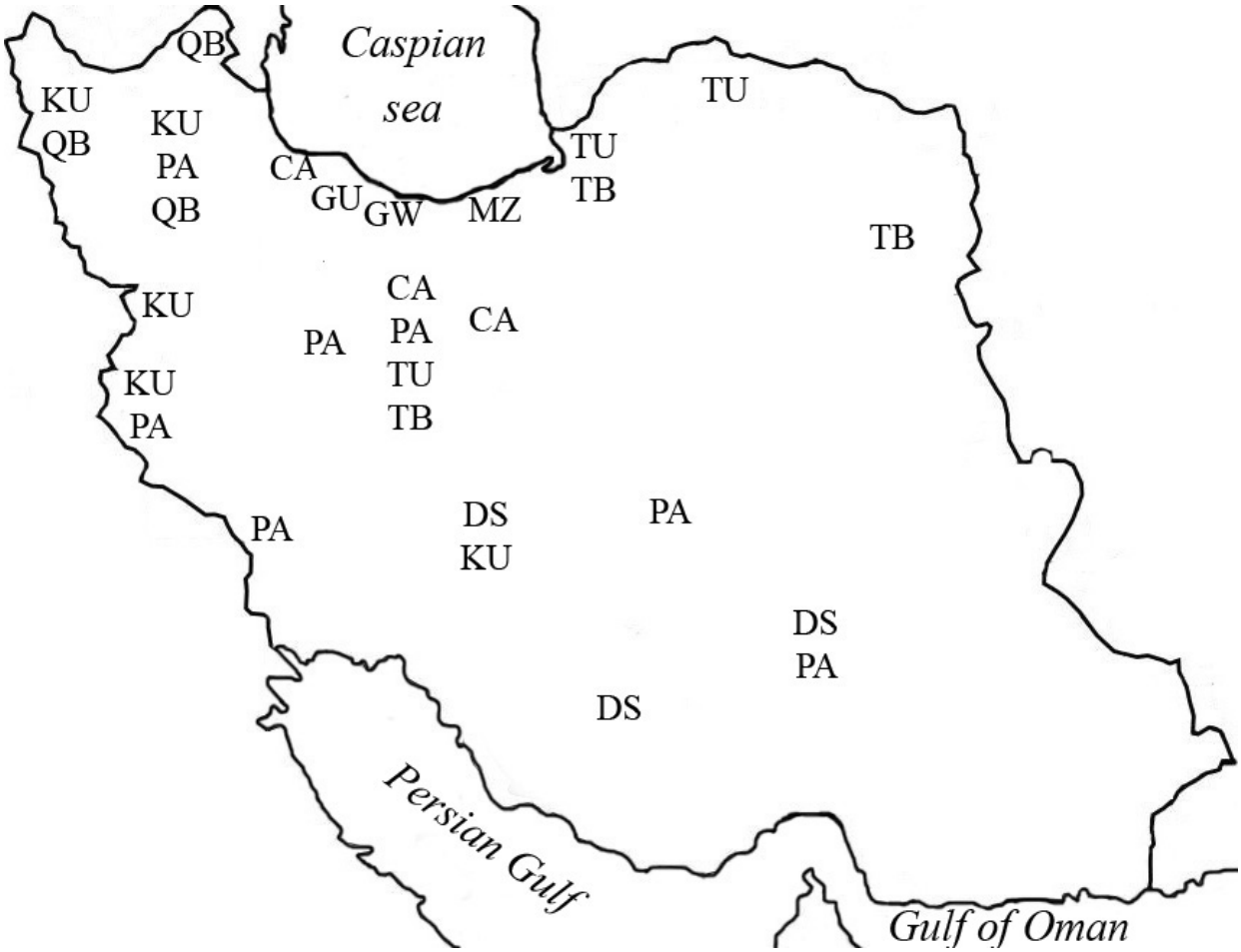

Figure 1. The geographical distribution of samples (CA: Caspian; DS: Qashqai (Dareh-Shoori); GU: Guilani; KU: Kurd; MZ: Mazandarani; PA: Persian Arab (Asil); QB: Qarehbagh; TU: Turkmen; TB: Thoroughbred; GW: Guilan wild horse).

Table 2. Details for the six Equus Y-specific microsatellite markers.

Microsatellite marker	Accession number	Primer (5' → 3')	Dye label	Annealing temperature (°C)
Eca.YA16	BV005729	F: TGACTGGAAATTGAAGATG R: TTGTAGCAACAAAGTAACAC	VIC	62
Eca.YE1	BV005727	F: CTTCACTCCCGACCAAGAGA R: GTGTGTCGTGCCGTGTTTAC	NED	62
Eca.YH12	BV005747	F: CGAACAGGTGACGAAGCATC R: GCAGACATGCACACCAACC	FAM	62
Eca.YJ10	BV005728	F: AGTTCCCCTGCACACCT R: TGCCTCCCACAGCCATAC	FAM	62
Eca.YM2	BV005725	F: TGGTTCAGATGGTGTATTTTGTT R: TTTGCAGCCAGTACCTACCTT	FAM	58
Eca.YP9	BV005726	F: AAGCACTGCCTTTTGGAATC R: AACCCTGGACTTTCTTTTGAA	NED	58

Table 3. Detected allele sizes (bp) for Y-specific microsatellites and Y Chromosomal haplotype frequencies in Iranian horse populations.

Parameter	Y-Specific microsatellite Loci					
	Eca.YA16	**Eca.YE1**	**Eca.YH12**	**Eca.YJ10**	**Eca.YM2**	**Eca.YP9**
Allele size (bp)	156	199	102	212	116	218
Haplotype frequency	1.00	1.00	1.00	1.00	1.00	1.00

chromosome may permanently circulate in the population (Santani, 2004).

The sex bias may be due to the breeding practices in Iran and most regions of the world in which a few selected stallions mate with many mares and therefore, the number of patrilines are reduced in population (Lindgren et al., 2004). For example, 80 stallions have contributed in current gene pool of thoroughbred horses, but just 10 of them have composed more than 50 percent of the genes in the whole population (Santani, 2004). Among these founders, three stallions (Darley Arabian, Godolphin Arabian and Byerley Turk) have played the main role. Moreover, in the current thoroughbred population, 95% of the males were descended from a single stallion, Darley Arabian (Cuningham et al., 2001). Hence, the number of males contributing to the next generations is very low which can reduce the genetic variation in Y chromosome. This breeding strategy was intensified with a strong tendency to upgrade many breeds by crossing Arabian and thoroughbred stallions to mares from different breeds, especially indigenous breeds (Wallner et al., 2004). In some cases, political forces have supported this strategy as well. For example, in Iran, King Nader Afshar (1735-1747 AD) forced a few Persian Arab stallions to migrate from Khuzestan (in Southwest of Iran) to Chenaran (in Northeast of Iran) for crossing in Turkmen mares which resulted into a multi-propose population named Chenarani (a subpopulation of Turkmen breed in Iran) (Tavakkolian, 1999). The low variation in Y-DNA is entirely in contrast with high variation in mtDNA (Jansen et al., 2006; Vila et al., 2001; Yang et al., 2002; McGahern et al., 2006; Kakoi et al., 2007). This implies that female breeding populations contributing to the gene pools of horse breeds were more prominent than that of the male populations. Such sex biased selection may be a commonplace practice among other domestic mammals. A prominent example of this practice is the case of dogs. Within dog breeds, fewer Y chromosome haplotypes than mtDNA haplotypes were found. This strongly indicates that a smaller number of males than females were involved in the formation of most dog breeds (Sundqvist et al., 2006). Using a limited number of stallions might be a traditional breeding practice which dates back to the initial steps of horse domestication (Lindgren et al., 2004). If a sex bias in breeding were only a modern phenomenon, it is expected to detect some Y chromosome variation within or among breeds. Lindgren et al. (2004) sequenced all Y chromosome fragments (14.3 bp) in a male Przwalski's horse and detected that it differed from all domestic horses at six nucleotides and deletion in 7 bp. Based on Y chromosome data, it is estimated that the separation between Przwalski's horse and domestic horses has occurred approximately 120,000 to 240,000 years ago (Wallner et al., 2003). Since this time is much older than the start of horse domestication era (approximately 6,000 years ago), thus wild horses may have had polymorphism in Y chromosome before domestication (Lindgren et al., 2004).

A sex bias towards males in the early utilization of horses could have played a role in the low male contributions to horse gene pool (Lindgren et al., 2004). The earliest record of horses dates back to Paleolithic age. The remains of several thousand horses were found around Solutré in France, which indicates that horses may have served as a part of human diet (Andrews and Legates, 1962). Food production is generally maximized if most males are consumed and females are left for reproduction (Lindgren et al., 2004). Therefore, this sex bias towards using males for human diet may have played a role in reducing male populations more than females. Archaeological records show that other domesticated males were preferentially hunted for food compared to females as well (Zader and Hesse, 2000; lau et al., 2008).

Moreover, in nomadic age of human life history, domestication may have been occurred in a restricted geographical region and therefore led to the incorporation of only a limited number of Y chromosome haplotypes into the breeding stocks. When human migrated to different regions, wild mares were captured from local populations and crossed with migrated stallions. Thus, the contrasting levels of genetic variation in mitochondrial DNA and Y-DNA seen in current horses reflect how the horse domes-tication events were distributed among early human societies (Lindgren et al., 2004).

Finally, it is necessary to point that in the two previous investigations in which Y-specific markers were isolated (Wallner et al., 2004; Lindgren et al., 2004), less than 1% of the horse Y chromosome was screened (Santani, 2004). This may not be representative of the genetic variation of the entire horse Y chromosome. In another study, 27 microsatellites were isolated by screening 25% of horse Y chromosome, but only one of them (not male specific) displayed polymorphism (Santani, 2004). Moreover, the number of samples in these studies was

very low. Wallner et al. (2004), Lindgren et al. (2004) and Santini (2004) analyzed 49 samples (of 32 breeds), 52 samples (of 15 breeds) and 33 samples (of 14 breeds), respectively. The low number of sample sizes may be a reason for finding no polymorphisms in these studies. Ling et al. (2010) used 531 samples of Chinese horse breeds and found polymorphisms in one of these markers. However, our results clearly displayed that these six horse Y-specific microsatellites are not sufficient to study genetic diversity of horse Y chromosome in Iranian indigenous horse breeds. It implies the necessity of providing a detailed map of the entire horse Y chromosome or at least the entire male specific region of it.

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