Donkey individual identification and parentage verification using a specifically designed 22plex PCR

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Introduction

Nowadays, donkeys and mules are essential for transportation in poor, arid, and rough regions around the world although, in developed countries, this pack animal is no longer required. As a consequence, not only individual breeds are endangered, but also the whole species is heading for extinction. One of the first stages in conservation program of endangered breeds is the evaluation of their genetic variability.

In this study we propose a multiplex PCR including a set of 22 microsatellite markers (AHT4, AHT5, ASB23, COR112, COR214, HMS2, HMS3, HMS5, HMS6, HMS7, HMS18, HTG6, HTG7, HTG10, HTG15, LEX27, LEX33, TKY297, THY312, TKY321, UCDEQ(CA)425 and VHL20), specifically designed for donkey, to be co-amplified simultaneously in a multiplex PCR. Primers sequences were re-designed to achieve a final configuration allowing all markers to be analyzed together.

The proposed microsatellites were all well-amplified in donkey samples and all of the microsatellites used in this study were amplified and were polymorphic in blood samples of domestic donkey breeds.

DNA Donkey Comparison Test 2016-2017 organized by International Society for Animal Genetics (ISAG) included these markers and propose a standardized nomenclature.

The mean allele number for the microsatellites included in the proposed panel is 5.70 using a small number of samples. The cumulative probability of exclusion reached 99.9997725%. This donkey-22plex PCR panel can be used for genetic characterization, parentage verification and individual identification.

Material & Methods

Sample Processing: Whole blood was collected in tubes containing Magic Buffer® or EDTA. Blood sampling was carried out by *Tecan® Genesis Freedom* allowing sample aliquoting and barcode reading.

DNA extraction was performed by *BioSprint 96 Blood Kit®* of *Qiagen®* in a 96 format.

PCR were performed with *Qiagen Multiplex PCR Kit®* by multipippetors from *Tecan®*. Amplification Program: 15' to 95ºC; 40 cycles of: 30'' to 95ºC, 45'' to 58ºC and 1' to 72ºC; 15' to 72ºC in a *GeneAmp® 9700 PCR System* of *Applied Biosystems®*.

PCR products were analyzed with an *ABIPrism 3130xl Genetic Analyzer®* from *Applied Biosystems®*. Results interpretation was performed by *GeneMapper®* software from *Applied Biosystems®*.

Donkey 22plex

Microsatellite	5' Modified Forward Primer	Reverse Primer	Chromosome in Horse	Range	Number of alleles	Concentration (µMolar)	H	PIC	PE(%)
VHL20	5' 6-FAM CAAGTCCTCTTACTTGAAGACTAG 3'	5' AACTCAGGGAGAATCTTCCTCAG 3'	20	72-86	3	0,042	0,41125	0,34481	18,3204%
AHT4	5' 6-FAM AACCGCCTGAGCAAGGAAGT 3'	5' GCTCCCAGAGAGTTTACCCT 3'	24	124-160	8	0,021	0,83250	0,81206	66,9046%
HMS7	5' 6-FAM CAGGAAACTCATGTTGATACCATC 3'	5' TGTTGTTGAAACATACCTTGACTGT 3'	1	160-184	7	0,028	0,54375	0,51447	33,9540%
LEX27	5' 6-FAM ACCACTGGGAAACTGTGTAA 3'	5' GCCCAGAATCCGAACC 3'	X	184-200	4	0,042	0,71875	0,66726	46,5604%
COR114	5' 6-FAM TCAAAATCCACACTCCCTTC 3'	5' TCCATAAAGAGTGGGACACTG 3'	-	210-250	6	0,042	0,57125	0,50487	31,1502%
HTG6	5' VIC CCTGCTTGGAGGCTGTGATAAGAT 3'	5' GTTCACTGAATGTCAAATTCTGCT 3'	15	72-98	4	0,021	0,62500	0,57809	38,3816%
TKY312	5' VIC GATCCTTCTTTTTATGGCTG 3'	5' AACCTGGGTTTCTGTTGTTG 3'	6	99-125	4	0,063	0,71625	0,66374	46,1148%
AHT5	5' VIC ACGGACACATCCCTGCCTGC 3'	5' GCAGGCTAAGGAGGCTCAGC 3'	8	124-156	11	0,063	0,85875	0,84421	72,1939%
HMS18	5' VIC CAACAATGAAAATTTGTCCTGTGC 3'	5' GTAAATGAGTAGACAATCATGAGG 3'	30	158-194	6	0,028	0,64750	0,61556	43,4857%
TKY297	5' VIC GTCTTTTTGTGCCTCTGGTG 3'	5' TCAGGGGACAGTGGCAGCAG 3'	1	214-236	6	0,084	0,77000	0,73404	55,5193%
COR112	5' VIC TTACCTGGTTATTGGTTATTTGG 3'	5' TCACCCACTAAATCTCAAATCC 3'	-	237-271	10	0,042	0,77750	0,75472	59,8397%
HTG10	5' NED CAATTCCCGCCCCACCCCCGGCA 3'	5' TTTTTATTCTGATCTGTCACATTT 3'	21	72-108	8	0,070	0,81625	0,79064	63,4377%
HTG15	5' NED TCCTGATGGCAGAGCCAGGATTTG 3'	5' AATGTCACCATGCGGCACATGACT 3'	5	120-142	4	0,028	0,67750	0,61015	39,4691%
ASB23	5' NED GCAAGGATGAAGAGGGCAGC 3'	5' CTGGTGGGTTAGATGAGAAGTC 3'	3	144-156	5	0,063	0,74625	0,70578	51,7618%
HMS6	5' NED ATGAAGCTGCCAGTATTCAACCATTG 3'	5' ATCTCCATCTTGTGAAGTGTAACTCA 3'	4	156-174	4	0,028	0,55375	0,46590	27,1336%
TKY321	5' NED TTGTTGGGTTTAGGTATGAAGG 3'	5' GTGTCAATGTGACTTCAAGAAC 3'	20	180-206	4	0,049	0,47875	0,44752	27,8729%
HMS2	5' NED CTTGCAGTCGAATGTGTATTAAATG 3'	5' ACGGTGGCAACTGCCAAGGAAG 3'	2	216-244	7	0,049	0,70250	0,66086	47,6400%
HMS5	5' PET TAGTGTATCCGTCAGAGTTCAAAG 3'	5' GCAAGGAAGTCAGACTCCTGGA 3'	-	90-116	4	0,049	0,30250	0,27949	15,4966%
HTG7	5' PET CCTGAAGCAGAACATCCCTCCTTG 3'	5' ATAAAGTGTCTGGGCAGAGCTGCT 3'	4	130-160	9	0,042	0,76500	0,74305	58,5051%
HMS3	5' PET ATATACCAACTCTTTGTCACATAACAAGA 3'	5' ATATACCATCCTCACTTTTTCACTTTGTT 3'	9	160-186	5	0,042	0,60750	0,54067	34,2868%
LEX33	5' PET TTTAATCAAAGGATTCAGTTG 3'	5' GGGACACTTTCTTTACTTTC 3'	4	190-220	4	0,056	0,49875	0,37437	18,7187%
CA425	5' PET AGCTGCCTCGTTAATTCA 3'	5' CTCATGTCCGCTTGTCTC 3'	28	228-256	4	0,049	0,59125	0,52402	32,4566%
				Total:	5,7				99,99977%

Table 1: Panel of donkey markers. Colors represent the dye used for each marker. It is also included the chromosome number were each one is located if it is known (in horse), allelic range and primer concentration on the PCR *multiplex* mix. Heterozygosity (H), Polymorphic Information Content (PIC) and Probability of Exclusion (PE) set out the usefulness of these markers to carry out paternity analysis. This panel can detect incorrect genealogical relationships with a Global Probability of Exclusion of 99.99977%.

Results

A *multiplex PCR* reaction, including 22 microsatellites markers, has been designed including most commonly used donkey-specific markers in laboratories around the world and included in the proposed list for the Donkey International Comparison Test 2016-2017 organized by the International Society for Animal Genetics (ISAG) (Table 1). In order to obtain higher probability of exclusion have set a procedure to amplify the largest number of markers as possible in a single PCR reaction. Particular concentration of each primer was very important to obtain a correct amplification of all markers in the multiplex PCR reaction. Robotic procedures were implemented to minimize the risk of genotyping errors and parentage verification mistakes when a large number of samples are analyzed.

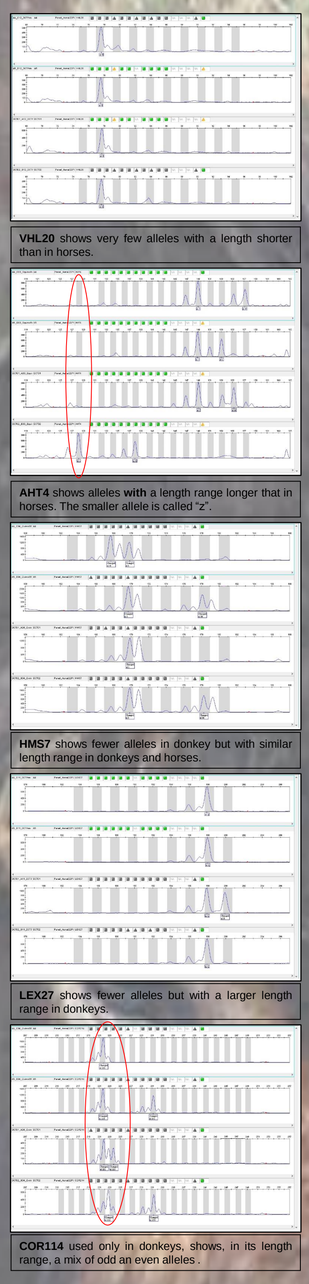
All of the microsatellites used in this study were amplified and were polymorphic samples of several domestic donkey breeds.

ISAG proposes to use the same allelic nomenclature, with letters, in horses and donkeys. It is important in mules (the offspring of a male donkey, a jack, and a female horse, a mare) or hinny (the offspring of a male horse, a stallion, and a female donkey, a jenny) were genotypes of horses and donkeys are used in the same paternity analysis.

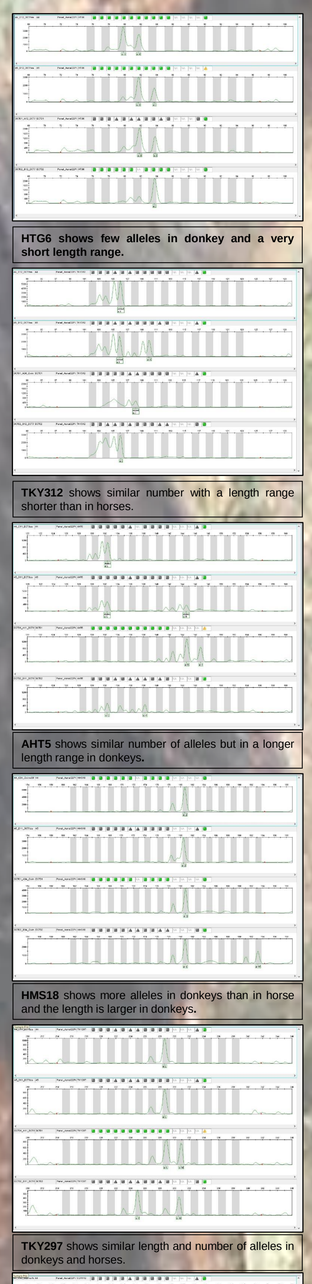
Some microsatellite markers (COR112 and COR114) show, in some donkey breeds, a mix of even and odds alleles. A correct interpretation of these alleles is important both in genealogical control and genetic characterization studies in order to avoid false compatibilities/incompatibilities or estimate the genetic diversity correctly. A mix of even and odd alleles is a problem when the names of the alleles are letters, this can be solved when numbers are used.

HMS3 is a known microsatellite marker with null o weak amplification alleles in practically all the horse breeds around the world but in the donkey breeds analyzed, until now in this laboratory, this type of allele seems not to be present.

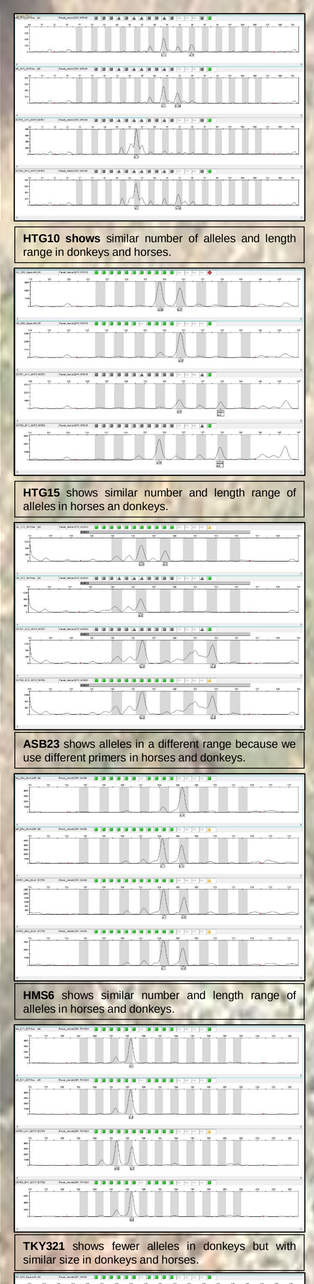
AHT4 shows alleles with a length range longer and shorter that in horses. The smaller allele is called "z" because is smaller than "A" in the allele denomination used internationally in horses.



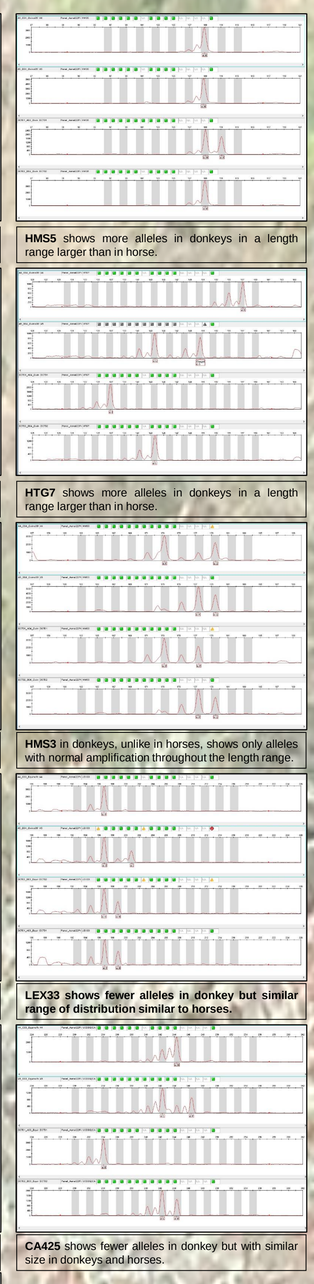
VHL20 shows very few alleles with a length shorter than in horses.



HTG6 shows few alleles in donkey and a very short length range.



HTG10 shows similar number of alleles and length range in donkeys and horses.



HMS5 shows more alleles in donkeys in a length range larger than in horse.

AHT4 shows alleles **with** a length range longer that in horses. The smaller allele is called "z".

TKY312 shows similar number with a length range shorter than in horses.

HTG15 shows similar number and length range of alleles in horses an donkeys.

HTG7 shows more alleles in donkeys in a length range larger than in horse.

HMS7 shows fewer alleles in donkey but with similar length range in donkeys and horses.

AHT5 shows similar number of alleles but in a longer length range in donkeys.

ASB23 shows alleles in a different range because we use different primers in horses and donkeys.

HMS3 in donkeys, unlike in horses, shows only alleles with normal amplification throughout the length range.

LEX27 shows fewer alleles but with a larger length range in donkeys.

HMS18 shows more alleles in donkeys than in horse and the length is larger in donkeys.

HMS6 shows similar number and length range of alleles in horses and donkeys.

LEX33 shows fewer alleles in donkey but similar range of distribution similar to horses.

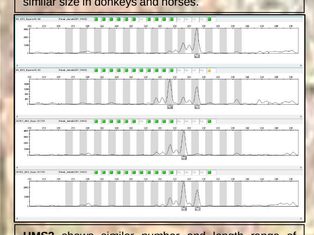
COR114 used only in donkeys, shows, in its length range, a mix of odd an even alleles .

TKY297 shows similar length and number of alleles in donkeys and horses.

TKY321 shows fewer alleles in donkeys but with similar size in donkeys and horses.

CA425 shows fewer alleles in donkey but with similar size in donkeys and horses.

COR112 used only in donkeys, shows, in its length range, a mix of odd an even alleles.



HMS2 shows similar number and length range of alleles in horses and donkeys

Discussion

High number of polymorphic markers amplified by PCR reaction is important to obtain a high probability of exclusion on paternity control. The proposed system allows to process a very large number of samples in a short period of time ensuring a accurate reliability and traceability of the obtained results.

Twenty two polymorphic microsatellite donkey-specific are amplified simultaneously in one multiplex PCR reaction. All the markers proposed are polymorphic and can be co-amplified in samples of several donkey breeds. This panel of markers can be used routinely for donkey parentage verification and reached and average number of alleles in these markers of 6.7 and a global probability of exclusion of 99.99977%.

The proposed panel of DNA markers can be used in several fields of animal breeding, e.g. examination of genetic structure of populations, estimation of populations inbreeding, maintenance of autochthonous populations, estimation of genetic distance between populations and breeds, as well as planning of crossing programs in endangered breeds.

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