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HETEROLOGOUS MARKERS IN FAMILY EQUIDAE

Vanshika Goel and Sainyam Goel
Indraprastha Convent Senior Secondary School
Begumpura, Rohini, New Delhi

INTRODUCTION

Equidae (The taxonomic family of horses and horse-related animals) include the extant horses, donkeys, zebras and other extinct species recognized only from fossils. Equines are odd toed ungulates with slender legs, long heads, relatively long necks, manes (erect in most subspecies) and long tails. All species are herbivorous, and mostly grazers with simpler digestive systems than ruminants but able to subsist on lower quality vegetation. On the basis of anatomy and DNA studies, there are three main divisions of Equidae (the horse family). 1. The caballines including a wild ancestor *Equus pizewalskii* and domestic horses. 2. The asses including African wild asses, hemionines and domestic donkeys. 3. Zebras. The only existing genus in the Equidae family is Equus. There are seven surviving species in the genus Equus (*Equus africanus*, *Equus ferus*, *Equus grevyi*, *Equus hemionus*, *Equus kiang*, *Equus quagga*, *Equus zebra*). The wild equids are found mainly in East Africa and near East to Mongolia. The domestic equids occur worldwide. The diploid chromosome number of Equus species vary from 32 of mountain zebra to 66 of przewalskii horse. (*Equus africanus asinus*) is a domesticated smallest equid of the Equidae family. African wild ass *Equus africanus* inhabiting arid and semi-arid bush/shrub land, grassland and desert is the ancestor of modern donkeys. Wild donkeys also known as burros reside in the desert plains. The hybrid animal produced by crossing of male donkey and female horse is known as mule and that of female donkey and male horse is known as hinny. The indigenous breeds of horses are Marwari, Kathiawari, Manipuri, Spiti, Bhutia and Zanskari. The Marwari is a rare horse breed belonging to Marwar (Jodhpur) of India. Marwari horse is known for its hardiness and peculiar lyre shaped ears that meet above the head to form an arch. Asses are believed exclusively of African origin. Nubian (North East African), Sudan (North East African) and Somali land (Somalian) were three wild races of ass. The present domesticated asses were predominantly descended from Nubian race. According to FAO reports, there are three different types of Indian asses: Indian, Indian wild and Kiang. Indian wild asses can be seen in Rann of Kutch whereas Kiang in Sikkim and Laddakh. Spiti ass belongs to Spiti and Lahaul of Himachal Pradesh and is used for transportation at high altitude where environmental oxygen level is low. This breed can tolerate the shortage of feed and harsh winter conditions.

The rapid decline in livestock diversity put our attention towards breed conservation strategies. The population with very low genetic diversity (variability of genes in a species) has lesser chance

to survive because it may not have characteristics required to tackle the new environmental pressure. Therefore the genetic diversity conservation is essential for the overall health of population because decreased genetic diversity results in inbreeding and diminished fitness. Evolutionary genetics is the broad field of studies that resulted from the integration of genetics and Darwinian evolution called the 'modern synthesis. This field attempts to account for evolution in terms of changes in gene and genotype frequencies within populations and the processes that convert the variation with populations into more or less permanent variation between equines. Some major tenets in evolutionary synthesis are that populations have genetic variations that arise by undirected processes (mutation and recombination). Some primary forces which bring about variations are genetic drift, gene flow and natural selection. Variation is the law of nature. Variations initiate at micro levels (bimolecular levels or genes) at short space and small time period but these become apparent only over a large space and with a time gap i.e. become apparent only at species and ecosystem level. Thus biological variation in nature over a time and space form the basis of evolutionary processes. To detect these variations which exist among individuals in the population in the specific regions of the genome, different molecular markers can be used.

Taxonomic Classification of horse

Horse (*Equus ferus caballus)* belongs to:

Kingdom	:	Animalia
Phylum	:	Chordata
Class	:	Mammalia
Order	:	Perissodactyla
Family	:	Equidae
Genus	:	*Equus*
Species	:	*E. ferus*
Subspecies	:	*E. f. caballus*

Taxonomic Classification of donkey

Donkey (*Equus africanus asinus*) belongs to:

Kingdom	:	Animalia
Phylum	:	Chordata

Class	:	Mammalia
Order	:	Perissodactyla
Family	:	Equidae
Genus	:	*Equus*
Subgenus	:	*Asinus*
Species	:	*E. africanus*
Subspecies	:	*E. a. asinus*

Microsatellites: - These are short sequences of di-or tri nucleotide repeats of variable length distributed widely throughout the genome. Using PCR primers to the unique sequences upstream and downstream of a microsatellite, their location and polymorphism can be determined and the technique is extensively used in investigating genetic association with disease.

The present work was carried out to find the homology of the two members of the family Equidae using a set of markers of horses which we amplified in both members of family equidae.

Experimentation:

A total of 96 blood samples were used for microsatellite analysis. Three populations (Marwari horses, Spiti donkeys and Barmer donkeys) were chosen in the present study. The blood samples of these animals were collected with the help of vacutainers and were brought from the field to the lab in ice packs.

Primer selection

The primers were selected on the basis of their distribution on the different chromosomes. The list of primers used for the present study of diversity analysis of Barmer donkeys, Spiti donkeys and Marwari horses are given in the table below.

Table 1. List of Primers.

Locus	Primer Forward	Primer Reverse
AHT04	AACCGCCTGAGCAAGGAAGT	CCCAGAGAGTTTACCCT
ASB02	CCACTAAGTGTCGTTTCAGAAGG	CACAACTGAGTTCTCTGATAGG

CORO22	CACCACGACTGCTTCCAATT	ACACACACACACACACACAC
CORO69	AGCCACCAGTCTGTTCTCTG	ACACACACACACACACACAC
CORO7	AGTTCTTCTGTGTGAGGCCA	CCCTCCAACTCCATACTCCC
CORO18	ACAGAAAGTGCCCTTGGTCA	TCCACTGCCACATCCTCAAT
HTG10	TTTTTATTCTGATCTGTCACATTT	CAATTCCCGCCCCACCCCCGGCA
VHL20	CAAGTCCTCTTACTTGAAGACTAG	AACTCAGGGAGAATCTTCCTCAG
AHT05	ACGGACACATCCCTGCCTGC	GCAGGCTAAGGAGGCTCAGC
ASB17	ACCATTCAGGATCTCCACCG	GAGGGCGGTACCTTTGTACC
CORO79	GGCTTTACATTTGGGGAGGG	CAGAAGTGGAGAGCGTGAAA
HMS06	GAAGCTGCCAGTATTCAACCATTG	CTCCATCTTGTGAAGTGTAACTCA
HTG06	GTTCACTGAATGTCAAATTCTGCT	CCTGCTTGGAGGCTGTGATAAGAT
LEX33	GGATTCAGTTGTGTGCGTGT	ACTTTCTCTTCAGGTGTCCTCA
NVHEQ54	TGTGCAGATGTCCACCTTCT	GACGGGGCTTTTAGGAGGTA
HMS03	CCATCCTCACTTTTTCACTTTGTT	CCAACTCTTTGTCACATAACAAGA
HTG07	CCTGAAGCAGAACATCCCTCCTTG	ATAAAGTGTCTGGGCAGAGCTGCT
SGCV28	CACCACGACTGCTTCCAATT	ACACACACACACACACACAC

Cocktail Preparation

Table 2 The cocktail for Multiplex PCR 96 reactions (1440 µl).

Sr. No.	Components	Stock concentration	Volume (µl)	Required concentration
1.	10X PCR buffer	10X	1.5×96 = 144	1X
2.	dNTPs	10mM each	0.3×96 = 28.8	200µM each
3.	Each Forward Primer	20µM	0.25×96 = 24.0	5pmol

4.	Each Reverse primer	20µM	0.25×96 = 24.0	5pmol
5.	*Taq* Polymerase	5U/µl	0.2×96 = 19.2	1U
6.	Distilled water		192.0 µl	
	Total		14,40µl	

2µl of template DNA was added directly in each well of the PCR plate. The PCR was carried out in Eppendorf thermo-cycler.

Table 3 PCR Program used for amplification.

Sr. No.	Steps	Temperature (°C)	Time period	No. of Cycles
1.	Initial denaturation	95	5 minutes	1 cycle
2.	Denaturation	94	45 sec	35 cycles
3.	Annealing	58	45 sec	
4.	Extension	72	1 minute	
5.	Final extension	72	10 minutes	1 cycle
6.	Final temperature	4	∞	

Agarose gel electrophoresis of amplified PCR products

After completion of the PCR program, the products were checked on 2% agarose gel for the amplification. Before loading into the well, gel-loading dye (Xylene cynol FF, Bromophenol blue in glycerol) was added to the sample and the samples were run under constant voltage conditions (100 V) till the two dyes were separated. Amplified product appeared as sharp bands in gel dock due to the intercalation of ethidium bromide.

Post-PCR dilution

PCR products were diluted for genotyping. 2µl PCR product from each well was mixed with 48µl distilled water.

PCR product : 2µl

Distilled water : 48µl

Total : 50µl

Genotyping reaction cocktail

Gene scan 500 LIZ size standard developed by Applied Biosystem was used for fragment sizing, LIZ size standard yields size fragments between 50 to 500bp providing 16 single standard labelled fragments of 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 bases. Each of the DNA fragments labelled with proprietary fluorophore, which results in a single peak

when run under denaturing conditions. Internal lane size standard was run with every sample for accurate sizing. The genotyping reaction components were:

Diluted PCR product	-	2.0µl
Hi-di Formamide	-	7.9µl
Liz size standard	-	0.1µl
Total	-	10µl

Denaturation

The above components were mixed well and denatured at 95°C for 5 min. The 96 well plate was loaded in automated DNA sequencer for genotyping.

Genotyping using automated DNA sequencer

Automated DNA sequencer **ABI 3130XL** (**Fig.8)** was provided with 16 capillaries with different array sizes (36cm, 50cm, 80cm). We perform genotyping using 36cm array size. The larger surface area of a capillary allowed heat generated during electrophoresis to be dissipated efficiently allowing high voltage electrophoresis. The result was rapid high-resolution separation of DNA fragments. Polymer POP7 (Performance Optimized Polymer) was used for sizing and separating of DNA fragments. Plate records were prepared and size standard was added to automated DNA sequencer prior to set up of the run and then 96 well plate was linked and started the run.

ABI 3130XL Automated DNA Sequencer

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Data collection and extraction

The electrophoresed data was extracted from automated DNA sequencer after sizing using Gene Scan software and the allele size were extracted using Gene Mapper software version 3.0. The data was exported as text file and imported into excel sheet before submitting it to further statistical analysis.

Statistical analysis of data

The data generated using the microsatellite loci were subjected to statistical analysis. The statistical analysis was carried out using Genealex, Arlequin, Genetix, Populations and Figtree softwares.

Results of experimentation

Donkeys and horses belong to the same family (Equidae), so there was a strong possibility for amplification of horse primers in donkeys. We tried to amplify the horse primers in donkeys. A total of 96 samples of domestic equines (Barmer donkeys, Spiti donkeys and Marwari horses) were taken and DNA was isolated. The sequence information about horse microsatellite loci was taken from HORSEMAP and NCBI. Many primers were designed for these microsatellite loci. 18 primers out of battery of primers amplified both in donkeys and horses. The Gene Mapper ver.3.0 software was used for extraction of genotypic data. The allele sizes of 18 loci were determined. The data extracted was suitably modified and run on several software (Genealex, Arlequin, Populations, Figtree and Genetix) to estimate gene and genetic diversity.

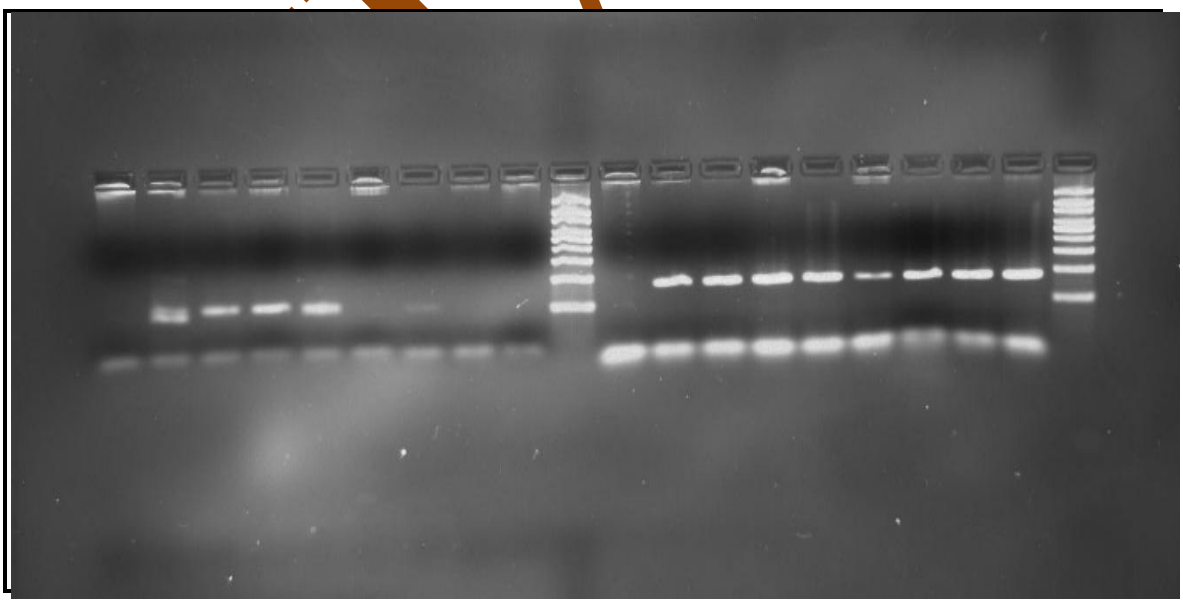

Fig.1. Bands on agarose gel after gradient PCR.

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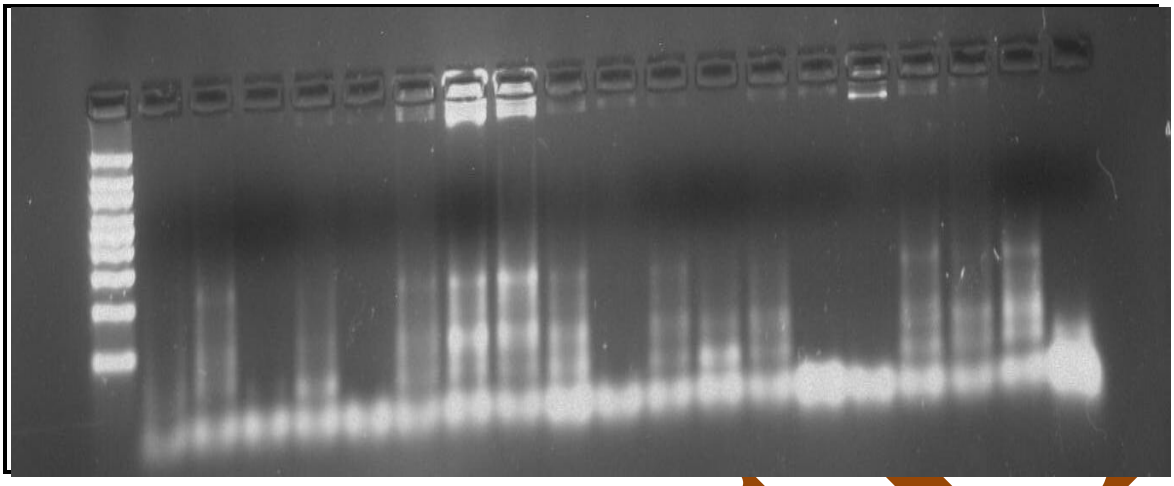

Fig.2. PCR product Bands on agarose gel after multiplex PCR.

Genetic characterization of Marwari horses, Spiti donkeys and Barmer donkeys

Number of alleles and allele size range

The amplified PCR products were sized using ABI 3130 automated sequencer with Liz 500 taken as internal size standard. The sizing of the alleles was found using Liz 500 internal size standard using Gene Mapper software (ver. 3.0). The number of alleles in each of the locus has been presented in the table. The number of alleles ranged between 2 in HTG07 in Spiti donkeys and 21 in CORO18 in Spiti donkeys. The mean number of alleles in 18 loci were found to be 8 in Barmer donkeys, 10.1 in Spiti donkeys and 12.2 in Marwari horses which signify that all the microsatellite loci taken in the present study were highly polymorphic in nature and could be used for association studies and diversity analysis of the populations.

Effective number of alleles and heterozygosity

The genetic variation in terms of number of alleles observed and effective number of alleles for each of the Barmer donkey, Spiti donkey and Marwari horse under each locus is given in the table. The observed heterozygosity (H_0) and expected heterozygosity (He) on the basis of allele frequency is also tabulated.

Table 4. Number of alleles observed, effective number of alleles, observed and expected heterozygosities of Barmer donkeys.

Barmer donkeys					
Locus	Na	Ne	I	Ho	He
AHT04	9.000	5.261	1.893	1.000	0.810

ASB02	5.000	3.315	1.327	0.909	0.698
CORO022	11.000	5.500	2.059	1.000	0.818
CORO069	9.000	3.507	1.703	0.818	0.715
CORO07	8.000	5.500	1.881	1.000	0.818
CORO18	15.000	11.000	2.563	1.000	0.909
HTG10	6.000	5.149	1.709	1.000	0.806
VHL20	9.000	4.939	1.870	0.909	0.798
AHT05	6.000	3.533	1.450	0.955	0.717
ASB17	10.000	6.769	2.069	0.773	0.852
CORO79	11.000	5.531	2.012	0.818	0.819
HMS06	5.000	2.350	1.019	0.955	0.574
HTG06	9.000	5.068	1.812	1.000	0.803
LEX33	6.000	2.734	1.216	0.955	0.634
NVHEQ54	6.000	5.762	1.772	1.000	0.826
HMS03	9.000	2.924	1.515	0.636	0.658
HTG07	6.000	1.333	0.611	0.227	0.250
SGCV28	4.000	1.205	0.399	0.182	0.170
Mean	8.000	4.521	1.604	0.841	0.704
SE	0.652	0.536	0.126	0.060	0.047

(where Na = Number of alleles, Ne = Number of effective alleles, Ho = Observed Heterozygosity, He = Expected Heterozygosity).

Table 5. Number of alleles observed, effective number of alleles, observed and expected heterozygosities of Spiti donkeys.

Spiti donkeys					
Locus	**Na**	**Ne**	**I**	**Ho**	**He**
AHT04	9.000	6.107	1.950	1.000	0.836
ASB02	8.000	3.390	1.504	0.950	0.705
CORO022	19.000	12.698	2.729	1.000	0.921
CORO069	11.000	3.053	1.677	0.750	0.673
CORO07	11.000	4.969	1.929	1.000	0.799
CORO18	21.000	11.429	2.774	1.000	0.913
HTG10	7.000	3.376	1.458	1.000	0.704
VHL20	8.000	4.082	1.621	0.950	0.755
AHT05	11.000	3.874	1.664	0.900	0.742
ASB17	9.000	4.885	1.800	0.875	0.795
CORO79	15.000	7.095	2.277	0.925	0.859

HMS06	8.000	2.428	1.117	0.925	0.588
HTG06	9.000	3.441	1.511	0.875	0.709
LEX33	14.000	5.565	1.987	0.975	0.820
NVHEQ54	9.000	4.113	1.629	1.000	0.757
HMS03	8.000	4.272	1.639	0.825	0.766
HTG07	2.000	1.078	0.160	0.025	0.072
SGCV28	3.000	1.253	0.391	0.025	0.202
Mean	10.111	4.839	1.657	0.833	0.701
SE	1.126	0.716	0.154	0.071	0.052

(where Na = Number of alleles, Ne = Number of effective alleles, Ho = Observed Heterozygosity, He = Expected Heterozygosity).

Table 6 . Number of alleles observed, effective number of alleles, observed and expected heterozygosities of Marwari horses.

Marwari horses					
Locus	**Na**	**Ne**	**I**	**Ho**	**He**
AHT04	11.000	7.293	2.123	0.964	0.863
ASB02	6.000	3.267	1.384	0.929	0.694
CORO22	20.000	12.748	2.749	1.000	0.922
CORO69	13.000	2.021	1.331	0.571	0.505
CORO7	16.000	6.426	2.245	1.000	0.844
CORO18	23.000	13.176	2.878	1.000	0.924
HTG10	12.000	6.374	2.106	1.000	0.843
VHL20	12.000	8.711	2.312	1.000	0.885
AHT05	10.000	4.785	1.828	0.938	0.791
ASB17	10.000	4.551	1.818	0.781	0.780
CORO79	16.000	9.706	2.543	0.813	0.897
HMS06	10.000	4.853	1.828	1.000	0.794
HTG06	11.000	5.185	1.911	0.875	0.807
LEX33	14.000	6.872	2.184	0.938	0.854
NVHEQ54	10.000	3.448	1.597	0.844	0.710
HMS03	10.000	3.556	1.761	0.688	0.719
HTG07	5.000	1.297	0.528	0.250	0.229
SGCV28	10.000	1.893	1.166	0.406	0.472
Mean	12.167	5.898	1.905	0.833	0.752
SE	1.042	0.811	0.136	0.052	0.043

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(where Na = Number of alleles, Ne = Number of effective alleles, Ho = Observed Heterozygosity, He = Expected Heterozygosity).

Allelic Patterns across Breeds/ species

The allelic pattern across the population is given in figure. The allelic patterns describes the number of alleles, number of effective alleles, number of private alleles, Shannon information index observed in each of 3 populations. The number of alleles with allele frequency greater than 5% have been shown separately. The effective number of alleles have been shown as Ne. The mean Ne in Barmer donkeys, Spiti donkeys and Marwari horses was found to be 4.521±0.536, 4.839±0.716 and 5.899±0.811 respectively. The Shannon information index which gives a measure of the heterozygosity and diversity has been depicted as I. The mean I in Barmer donkeys, Spiti donkeys and Marwari horses was found to be 1.604±0.126, 1.657±0.154 and 1.905±0.136 respectively. The number of alleles which are private to separate populations are shown above. The mean number of private alleles in Barmer donkeys, Spiti donkeys and Marwari horses were found to be 0.833±0.259, 1.056±0.249 and 3.444±0.493.

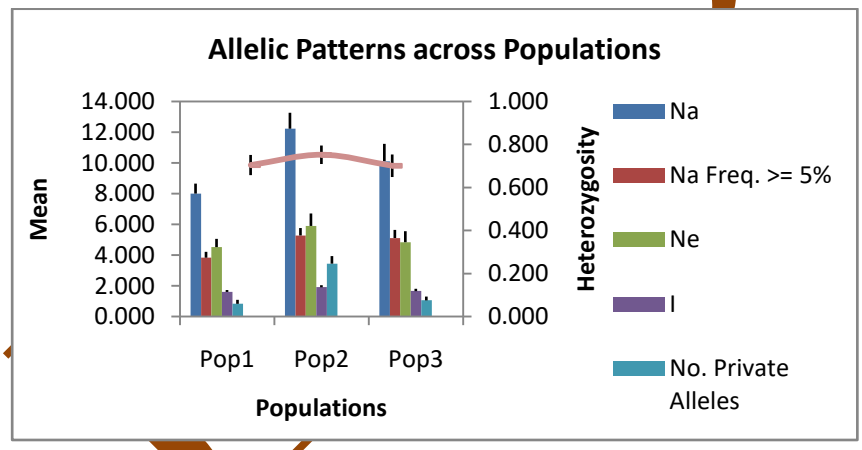
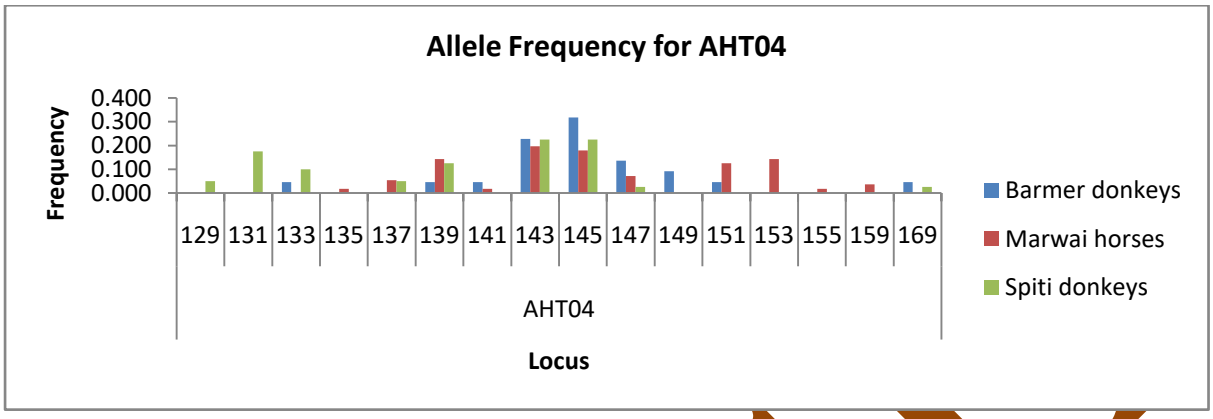
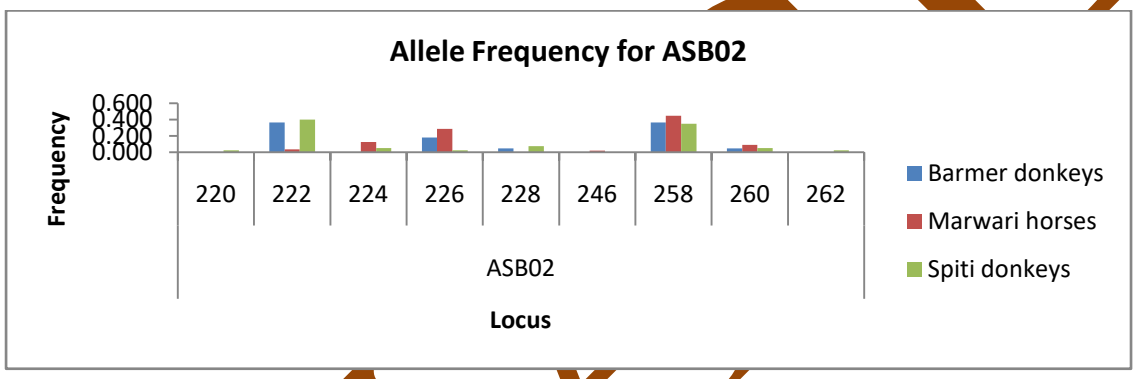
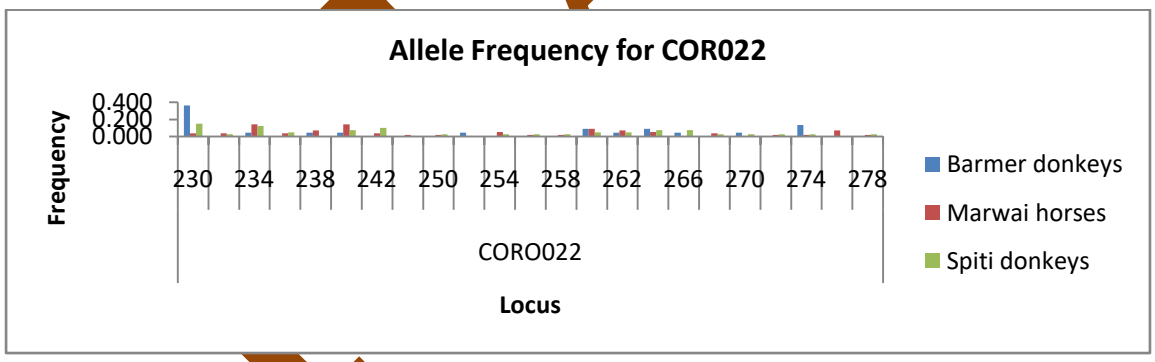


Fig.3. Graphical representation of allelic patterns across populations.

Diagramatic representation of allele frequency data







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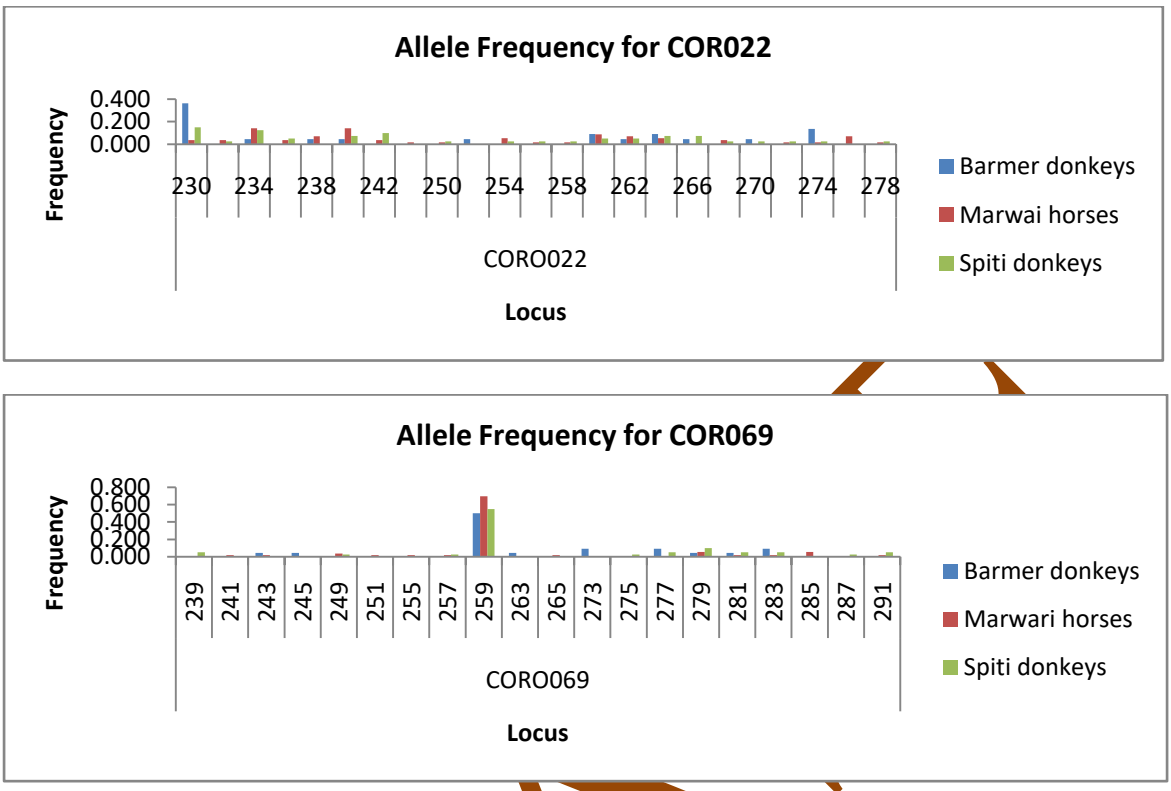
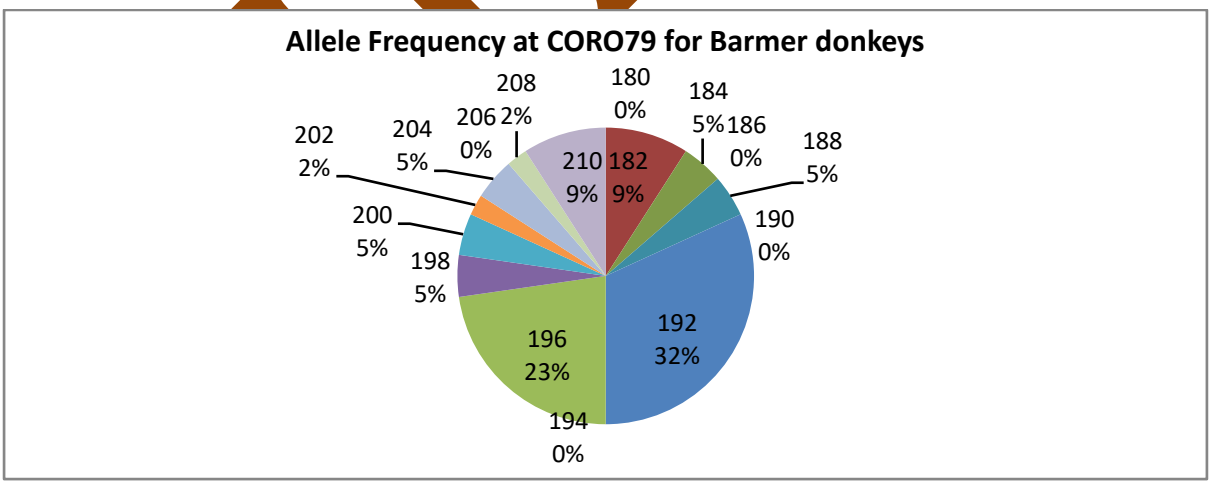
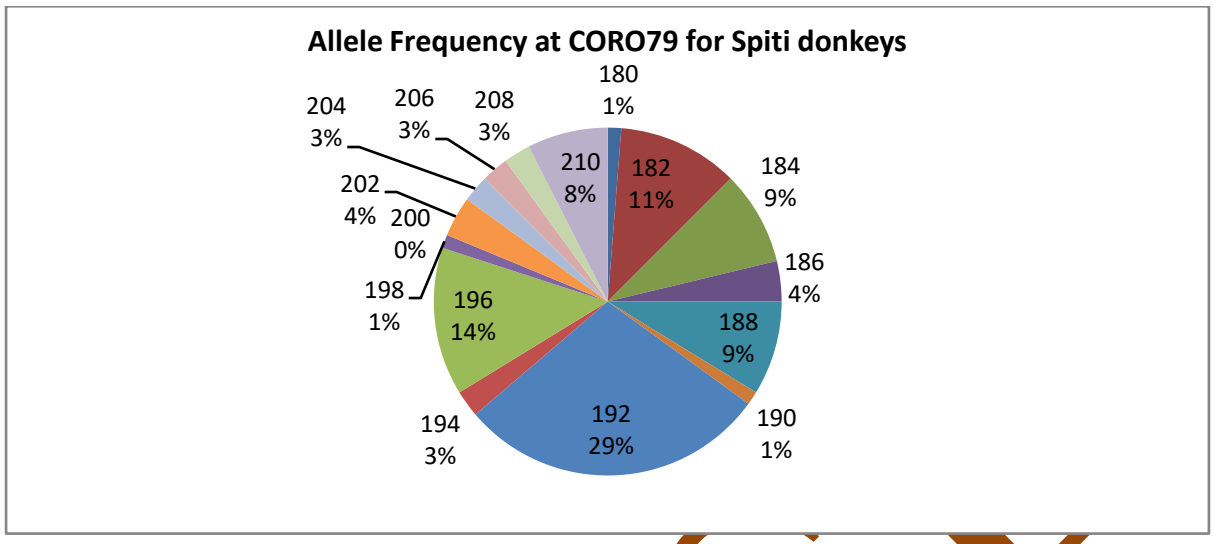
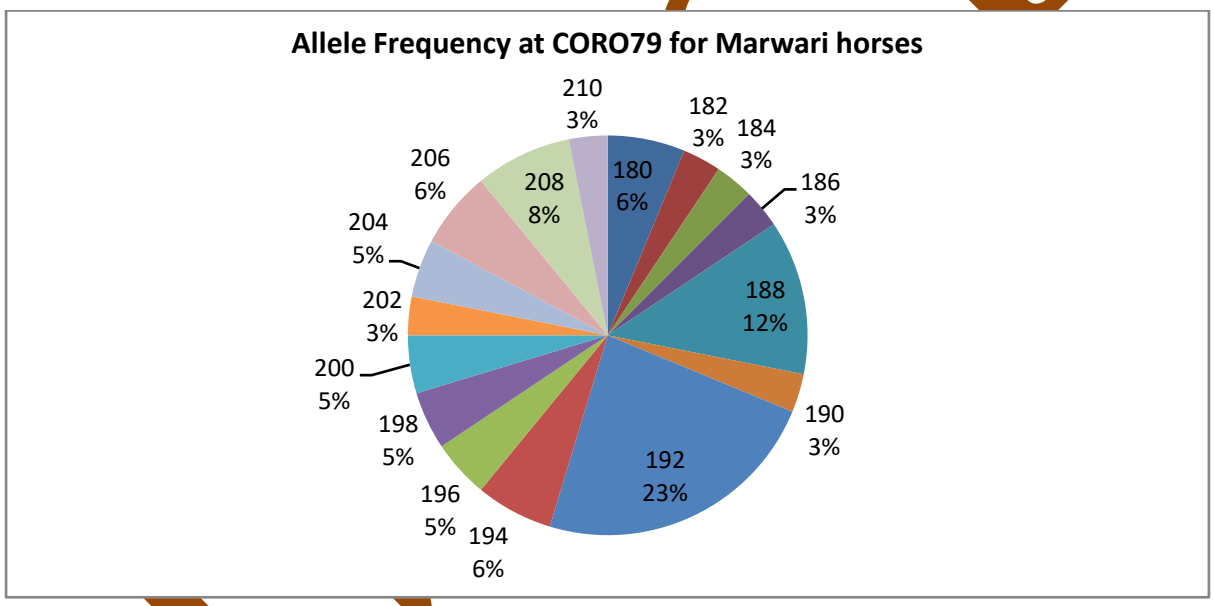


Fig 4.. Graphical representation of Allelic frequencies for representative loci in Barmer donkeys, Spiti donkeys and Marwari horses.




Allele Frequency at CORO79 for Spiti donkeys
180 1%
182 11%
184 9%
186 4%
188 9%
190 1%
192 29%
194 3%
196 14%
198 1%
200 0%
202 4%
204 3%
206 3%
208 3%
210 8%


Allele Frequency at CORO79 for Marwari horses
180 6%
182 3%
184 3%
186 3%
188 12%
190 3%
192 23%
194 6%
196 5%
198 5%
200 5%
202 3%
204 5%
206 6%
208 8%
210 3%

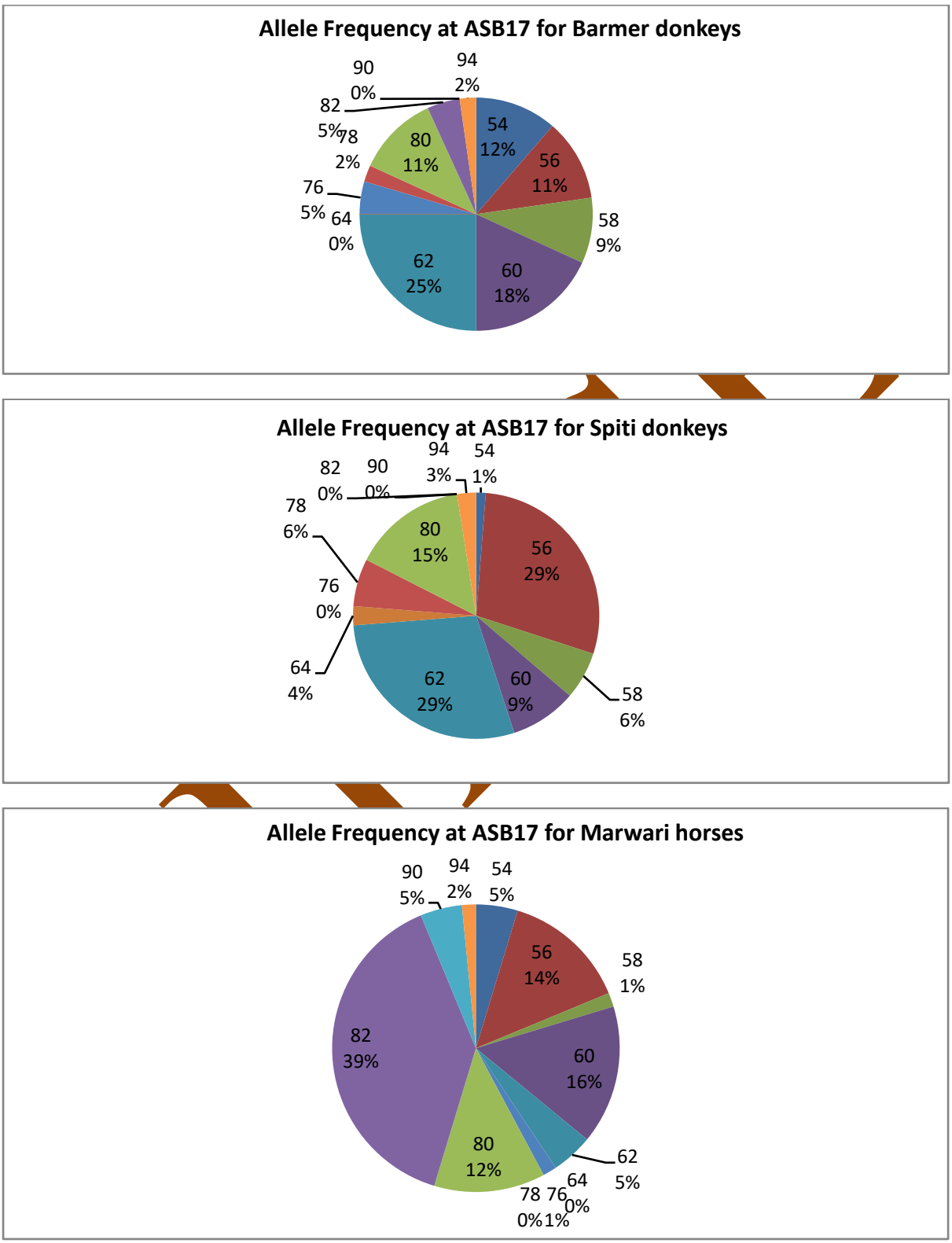

Allele Frequency at ASB17 for Barmer donkeys
90 0%
94 2%
82 5%
78 2%
80 11%
54 12%
56 11%
76 5%
64 0%
58 9%
62 25%
60 18%
Allele Frequency at ASB17 for Spiti donkeys
94 3%
54 1%
82 0%
90 0%
78 6%
80 15%
56 29%
76 0%
64 4%
62 29%
60 9%
58 6%
Allele Frequency at ASB17 for Marwari horses
90 5%
94 2%
54 5%
56 14%
58 1%
82 39%
60 16%
80 12%
62 5%
64 0%
78 0%
76 1%

Fig. 5. Pie-graphical representation of allele frequencies of 4 loci in Barmer donkeys, Spiti donkeys and Marwari horses.

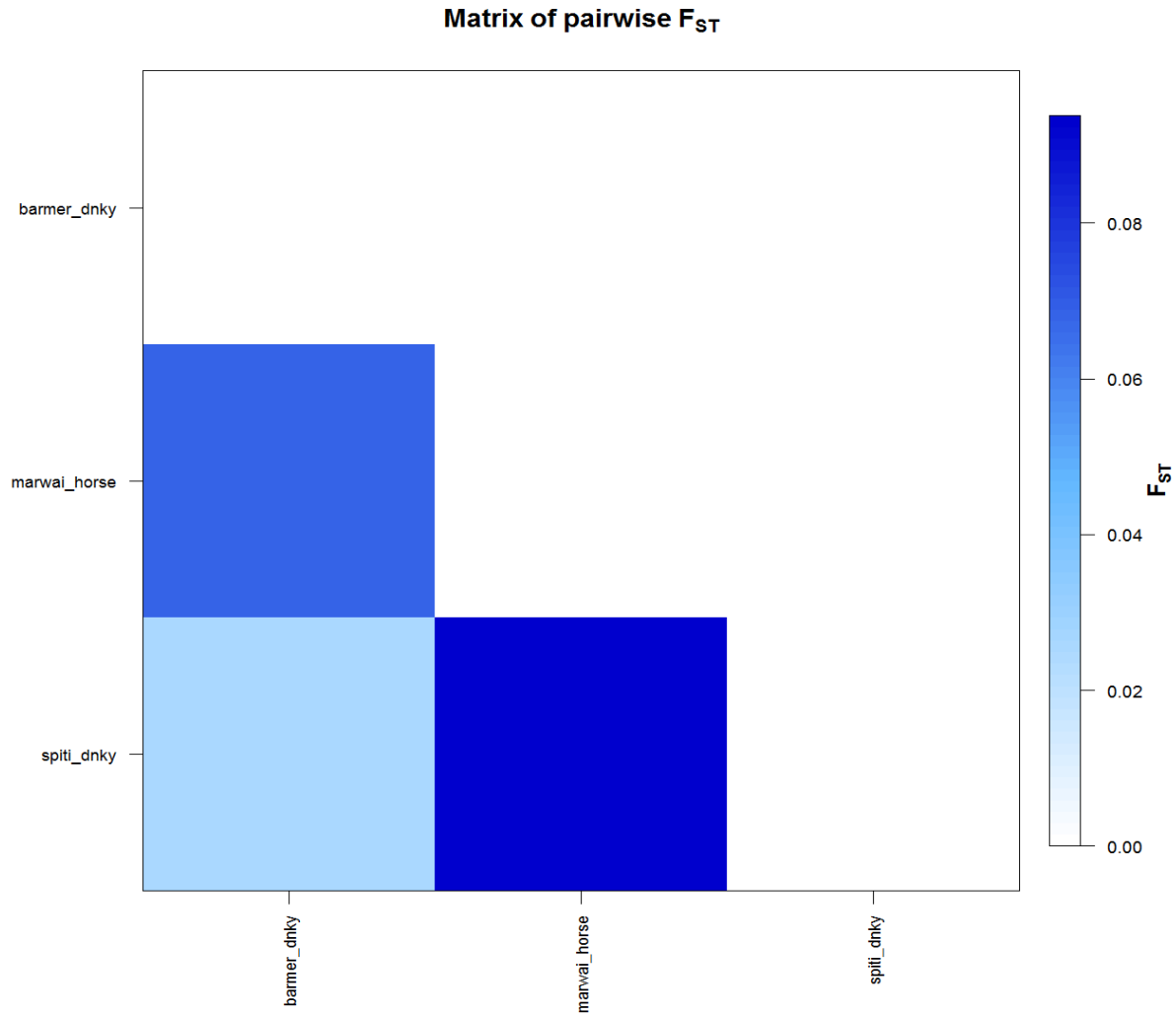


Fig 6 Pairwise F_{ST} and number of migrants (Nm)

F_{ST} gives the values of population differentiation while values of Nm depict the effective number of migrants per generation on the basis of f-statistics. A value beyond 1.0 means substantial migration. The estimates of F_{ST} revealed that Marwari horses are more differentiated than Spiti and Barmer donkeys. The graphical analysis revealed the distinction of Marwari horses from Spiti and Barmer donkeys.

Graphical representation of Pairwise F_{ST} of Spiti donkeys, Barmer donkeys and Marwari horses.

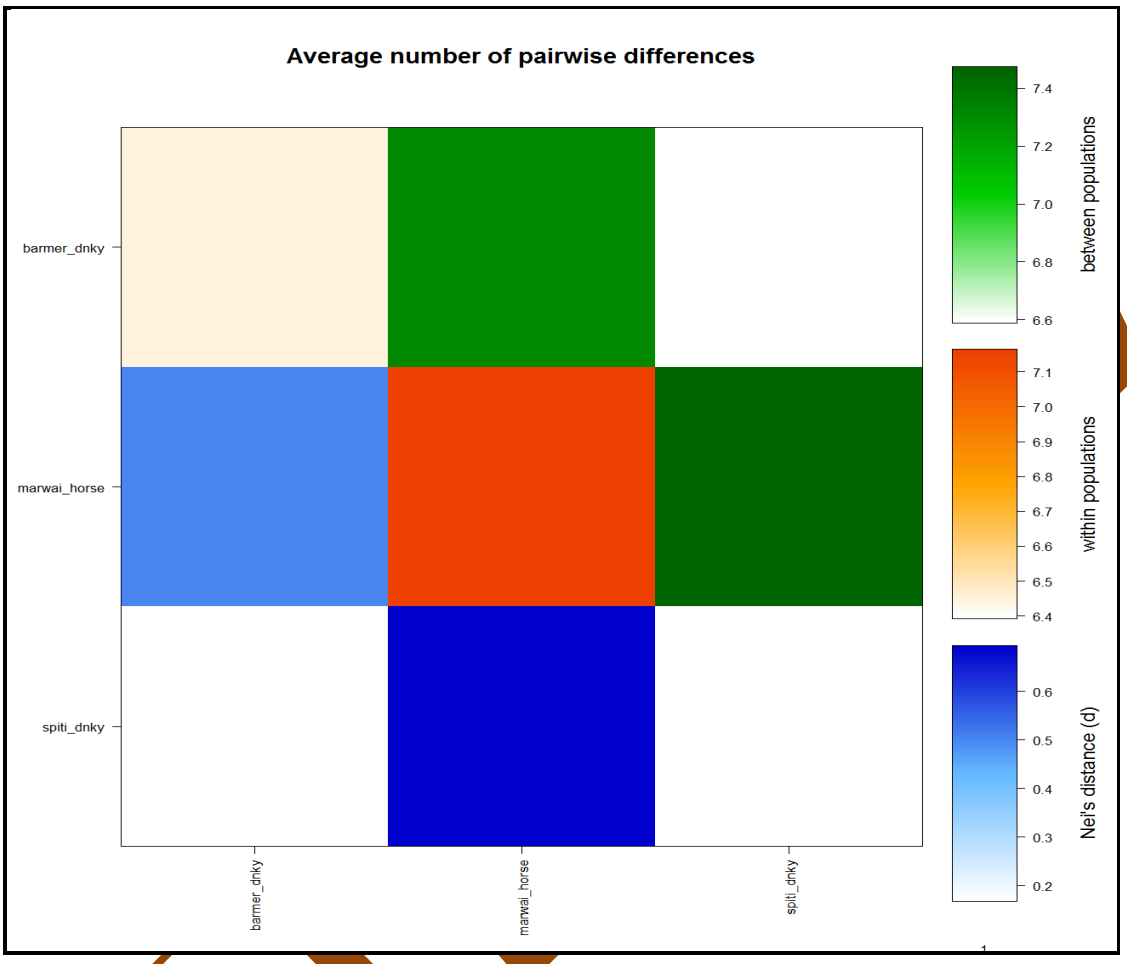


Fig 7. Graphical representation of Average number of pairwise differences.

Genetic Distance

The Nei's genetic distance is basically a correlation among the allele frequencies between the populations. The Nei's genetic distance and genetic identity is given in table below:

Correspondence analysis

The correspondence analysis was carried out using GENETIX ver. 4.05. The correspondence analysis was applied on multi-locus genotypes to explore the distribution of genetic variation graphically in a manner that animals with similar genotypes tend to cluster together. The

correspondence analysis of each of Barmer donkey, Spiti donkey and Marwari horse is shown in 3D view

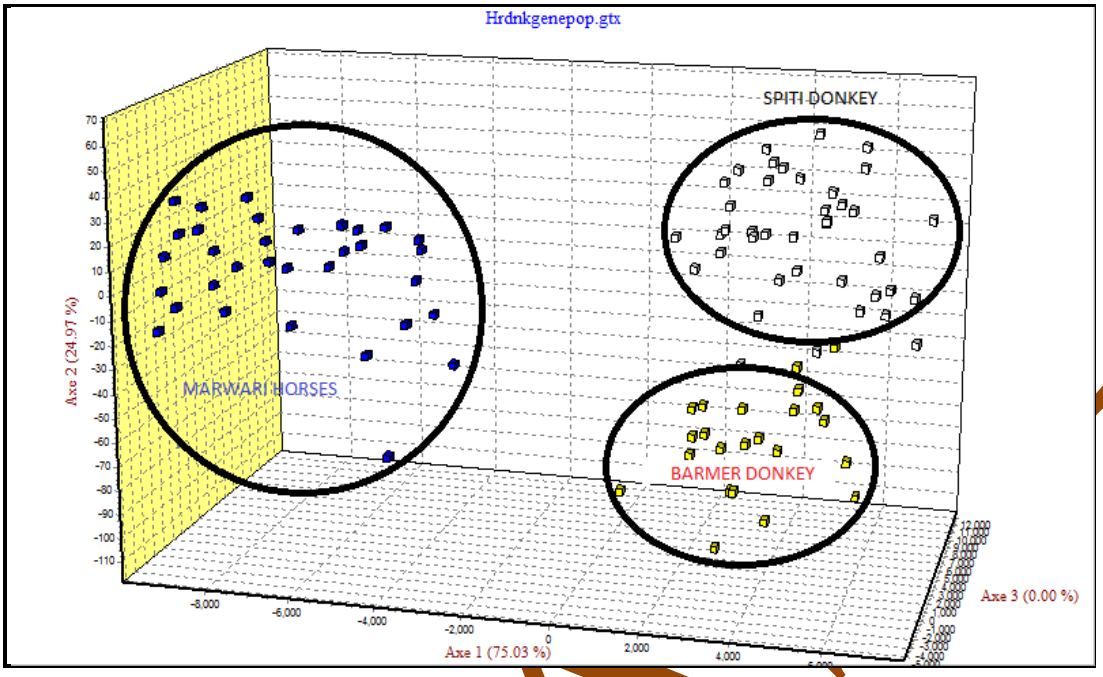


Fig 8. Correspondence analysis of Marwari horses, Spiti donkeys and Barmer donkeys (where position of each animal is shown separately).

The correspondence analysis reveals that Marwari horses clustered separately because of species difference. On the other hand, Spiti and Barmer donkeys clustered close to each other. This is because these belong to the same species.

Population Assignment

The population assignment was done for Barmer donkeys, Spiti donkeys and Marwari horses. The data of Population Assignments for all 3 populations is given in graphical representation and shown in fig. below:

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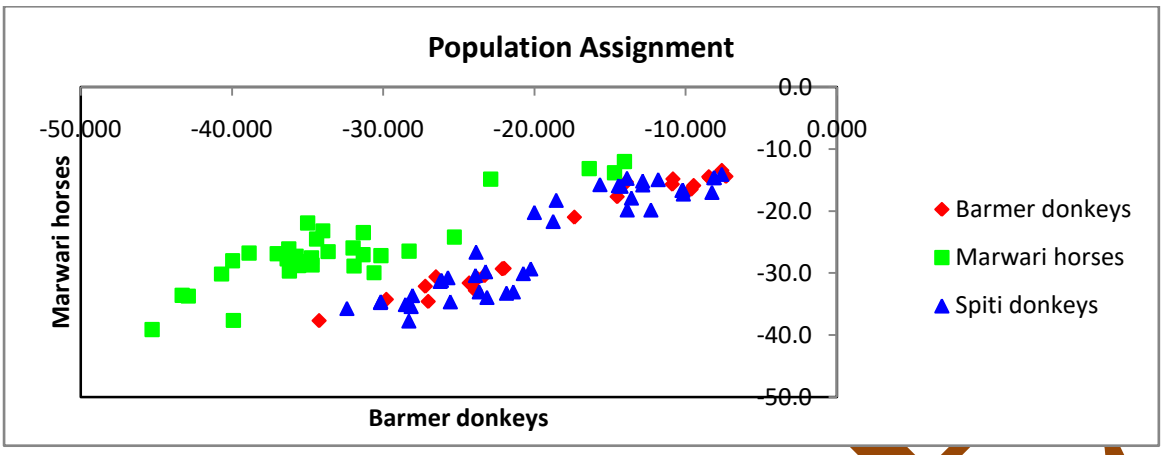

Fig 9. Graphical representation of Population Assignment in Barmer donkeys, Spiti donkeys and Marwari horses.

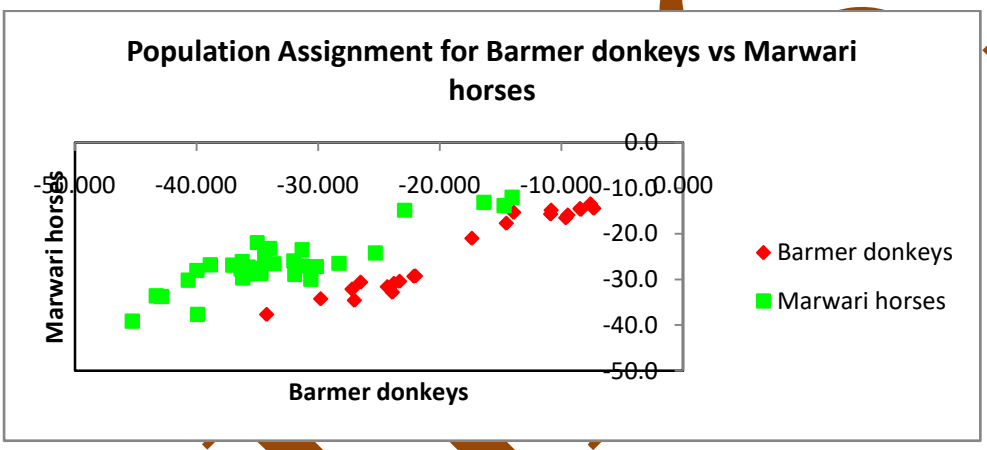

Fig 10. Population Assignment for Barmer donkeys vs Marwari horses.

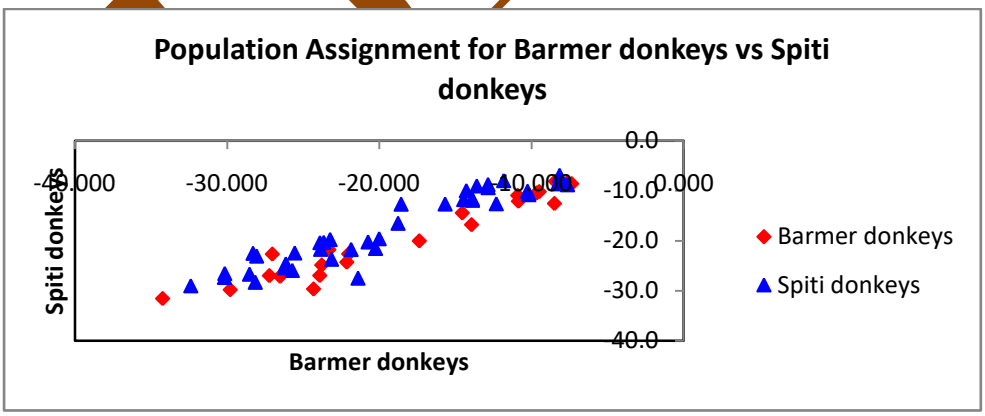

Fig 11. Population Assignment for Barmer donkeys vs Spiti dokeys.

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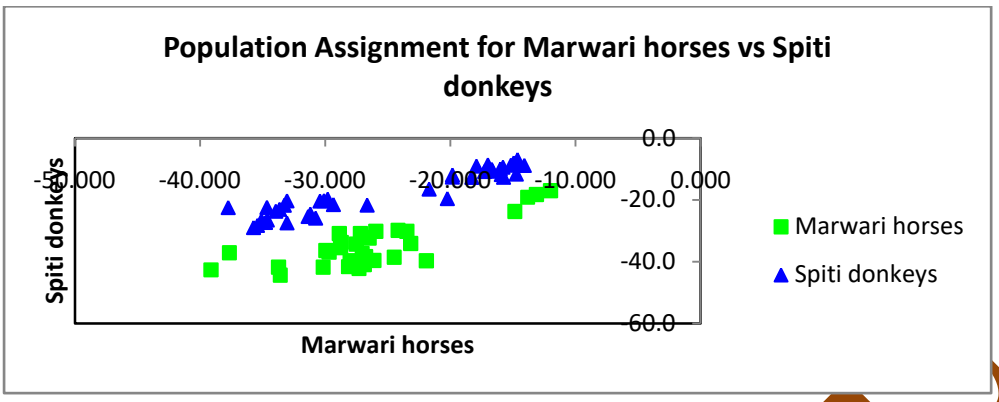

Fig 12.Population Assignment for Marwari horses vs Spiti donkeys.

Percentage and number of individuals belonging to self and other population for Barmer donkeys, Spiti donkeys and Marwari horses.

Populations	Self Pop	Other Pop
Barmer donkeys	16	6
Marwari horses	31	1
Spiti donkeys	30	10
Total	77	17
Percent	82%	18%

The data of population assignment shows that the animals in our study show 82% assignment to their population while 18% animals were assigned to same species populations. This is due to the same family of all the 3 populations. One wrong assignment could be due to it being mule.

Phylogenetic tree

A **phylogenetic tree** or **evolutionary tree** is a branching diagram or "**tree**" showing the inferred **evolutionary** relationships among various biological species or other entities—their **phylogeny**—based upon similarities and differences in their physical or genetic characteristics.

The phylogenetic trees shown below were drawn on the basis of Nei's inter-individual genetic distance. The trees reveal that the Barmer donkeys and Spiti donkeys are close to each other whereas Marwari horses have more genetic distances with both of donkey populations.

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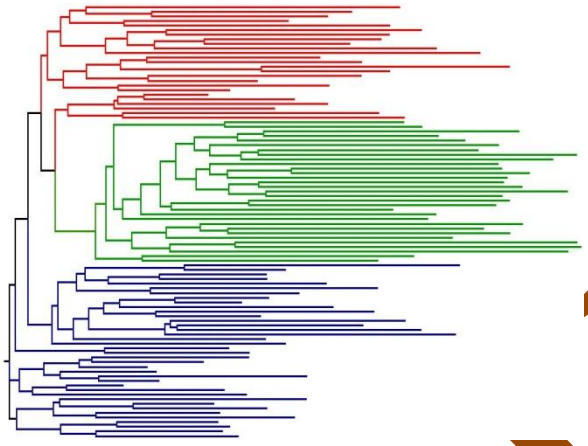

Dendrogram showing Inter Individual genetic relationship among Marwari horses, Spiti donkeys and Barmer donkeys (red – Barmer donkeys, blue – Spiti donkeys and green – Marwari horses obtained by distance based on allele sharing .

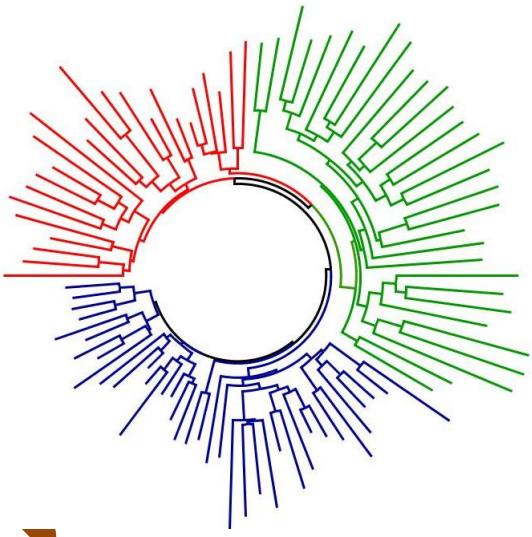

Fig 13 Circle tree constructed from Distance based on Allele Sharing inter individual genetic distance utilising neighbour-joining algorithm Marwari horses, Spiti donkeys and Barmer donkeys (red – Barmer donkeys, blue – Spiti donkeys and green – Marwari horses).

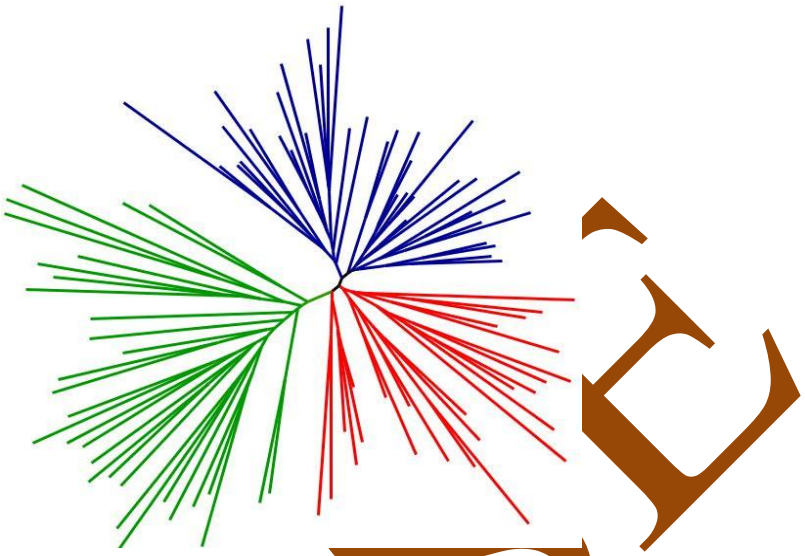

Fig 14. Neighbour-joining tree based on Nei's inter-individual genetic distances (red– Barmer donkeys, blue – Spiti donkeys and green – Marwari horses).

SOME INTERPRETATIONS:

1. The present study compared two breeds of donkeys and one breed of horse. Same markers were utilised for the study. The two species belong to the same family equidae. The two species are evolutionarily related to one another.
2. The Barmer donkeys have their breeding tract in Rajasthan while the Spiti donkeys are inhabitants of Spiti valley of Himachal Pradesh. The only horse breed taken for the study belongs to Rajasthan.
3. The study included use of heterologus markers in the two species of family equidae. The microsatellite were selected from the horse genome data base . The microsatellite loci amplified in both donkeys and horses. The cross amplification of the horse microsatellites in donkey confirm the usage of heterologus markers in the two species belonging to same family. The two species are evolutionary related not only to one another in morphology but also at DNA sequence level.
4. The 18 microsatelltie loci taken in the present study were polymorphic in both the species viz; horses and donkeys.
5. All the 18 microsatellite loci were polymorphic in Barmer donkeys. The mean number of alleles in Barmer donkeys were 8.0 ±0.652. The highest number of allele were obtained were 15 alleles for locus CORO18 while the lowest were 4 alleles for locus SGCV 28.

6. The estimated effective number of alleles were 4.521 ± 0.536. The effective number of alleles were almost half the total number of alleles observed. This reveals that almost half the number of alleles are at low frequency in the population and may be lost from the population by chance.
7. All the 18 microsatellite loci were polymorphic in Spiti donkeys. The mean number of alleles in Spiti donkeys were 10.11 ±0.652. The highest number of allele obtained were 21 alleles for locus CORO18 while the lowest were 3 alleles for locus SGCV 28. The estimated effective number of alleles were 4.839 ± 0.536.
8. All the 18 microsatellite loci were polymorphic in Marwari horses. The mean number of alleles in Marwari horses were 12.167 ±1.042. The highest number of allele were obtained were 23 alleles for locus CORO18 while the lowest were 5 alleles for locus HTG 07. The estimated effective number of alleles were 5.898 ± 0.811. The effective number of alleles were almost half the total number of alleles observed. This reveals that almost half the number of alleles are at low frequency in the population and may be lost from the population by chance.
9. The heterozygosity values obtained for the two populations of donkeys and one population of horses was quite high. The observed heterozygosity in Barmer donkeys was found to be 0.841 ± 0.060. Out of 18 loci seven loci exhibited the heterozygosity values to be 1.00. The mean expected heterozygosity of the 18 loci has been found to be 0.704 ± 0.047. The higher values of observed heterozygosity compared to expected heterozygosity points towards lack of population structure but also points to large number of migrants and subsequent geneflow among donkey populations.
10. Out of 18 loci six loci exhibited the heterozygosity values to be 1.00 in Spiti donkeys. The mean heterozygosity of the 18 loci has been found to be 0.833 ± 0.071 and the expected heterozygosity values of 0.701 ± 0.052. The higher values of observed heterozygosity compared to expected heterozygosity points towards lack of population structure but also points to large number of migrants and subsequent gene flow among donkey populations.
11. Six loci exhibited the heterozygosity values to be 1.00 in Marwari horses. The mean heterozygosity of the 18 loci has been found to be 0.833 ± 0.052 and the expected heterozygosity values of 0.752 ± 0.043. The higher values of observed heterozygosity compared to expected heterozygosity points towards lack of population structure but also points to large number of migrants and subsequent gene flow among different horse breeds.
12. The higher Nm values obtained in the study point to non existence of population structure and large number of migrants detected in the study. The values obtained was close to 7.0 (6.996) and thus the populations are not structured and the effective number of migrants per generation seem to be quite high among donkey populations.

13. High values of Fst obtained in the analysis point to the very large difference between the horse and donkey populations. While the Fst values are small between the two donkey populations viz Spiti and Barmeri.
14. Similarly the another genetic distance measure utilised in the present study was Nei's Standard genetic distance. The Nei's genetic distance between the horse and the two donkey populations was high viz; 0.254 and 0.304 for Barmer and Spiti donkeys respectively. The Nei's standard genetic distance was small among the donkey populations and was 0.094 which point to the existence of very similar genotypes and gene frequencies at the 18 loci studied.
15. The multivariate analysis of the 18 microsatellite data using Correspondence analysis also revealed that in a 3 dimensional figure the two donkey populations are closer to one another while the Horse is a distinctive 3rd.
16. The population assignment test revealed that out of 22 Barmer donkeys were 16 were assigned correctly. The results were quite good for Marwari horses in which out of a total of 32 horses 31 were correctly assigned. In case of Spiti donkeys out of a total of 40 animals 30 were correctly assigned while 10 were assigned to other donkey population. The results emphasize that the difference between the two donkey populations was not very high leading to wrong assignments while the horse was distinctive leading to high assignment accuracy.

Thus the present study has provided an insight on the diversity of the family equidae, several demographic parameters have been assessed and genetic distances estimated. The deviations of the populations in terms of Hardy Weinberg equilibrium and also the Mutation drift equilibrium has also been tested. The data suggests the usefulness of horse microsatellites for their use in donkeys.

The study thus help in diversity analysis of Indian donkey populations for their conservation as their number is declining at a faster rate due to gradual erosion of their importance as pack animals.

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