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Simple sequence repeat (SSR) genotypic data reveal high genetic diversity in Rajasthan donkey of India

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ABSTRACT

Spiti is the only breed of donkey in the list of 160 breeds registered so far for the indigenous germplasm. It points towards the fact that Indian donkey diversity is largely unexplored, in spite of its role in support system of poorest of the poor countrymen. Rajasthan state harbours more than eighty thousand donkey. Genetic diversity underpins population resilience and persistence so a need was felt for the systematic and scientific evaluation of existing diversity in this population. The diversity status of Rajasthan donkey population was investigated by using 20 microsatellite markers. Two of the loci, HMS5 and ASB17, depicted less than 4 alleles and were thus excluded from further analyses. Rajasthan donkey was found to host considerable diversity. This conclusion is evidenced by the number of alleles observed across loci (average 8.16±0.89), and by mean observed heterozygosity (0.688±0.06). Signatures of inbreeding could not be detected by the F_{IS} indices in the Rajasthan donkey population. The population did not suffer any bottleneck in the recent past. The study provides the first scientific assessment of the genetic diversity status of Rajasthan donkey population. This information on the genetic analysis can be used in conjunction with the information on physical and phenotypic characteristics and management practices for registration of this population as a breed.

Key words: Donkey, Genetic characterization, Microsatellite, Rajasthan

The donkey (*Equus asinus*) was domesticated more than 6,000 years ago proceeding from one or two subspecies of African wild asses (*E. africanus*) (Rossel *et al.* 2008). For many centuries, donkey has been used as beast of burden playing important role in transportation (Pal *et al.* 2013).The world population of this weight bearing mammal is more than 40 million and is still steadily growing (FAO 2014). China alone has around 11 million donkeys, closely followed by Ethiopia and Mexico. However, the trend is reverse in our country. According to the 19th Livestock census of India, there are 0.32 million donkeys as of 2012, a decrease of 27% compared to the previous census in 2007 (Anonymous 2017a). This can be attributed to the agricultural mechanization and economic development of the India. With respect to the characterization of different populations in the country, donkey has remained a neglected species (Behl *et al.* 2011). It is reflected in the official records too. Total number of indigenous breeds in the country is 160, which include 40 for cattle, 13 for buffalo, 26 for goat, 42 for sheep, 6 for horses and ponies, 9 for camel, 6 for pig, 17 for chicken and only1 (Spiti) for donkey (http://www.nbagr.res.in). Therefore, the need of characterization for this valuable genetic resource is urgent, and assessing the genetic diversity and relationships between and within donkey populations is a critical step in this process. One such population is found in the Rajasthan state of India.

The investigations on genetic diversity of donkey breeds have been limited in India as well as across the globe. Genetic characterization of donkey population is required for a basic understanding of the variation existing within the population. Molecular markers, especially simple sequence repeat (SSR) or microsatellites are valuable tools used to evaluate genetic diversity and relationships within and between animal breeds. High degree of polymorphism exhibited by the microsatellites markers make them extremely informative for diversity analysis and provides very high discriminating power (Seilsuth *et al.* 2016, Sharma *et al.* 2015). Microsatellite markers have been extensively used to establish donkey diversity status due to their random distribution across the genome, co-dominance, possibility of automated scoring of genotypes and neutrality with respect to selection. Jordana *et al.* (2001) found that microsatellite loci are effective at revealing genetic variation among donkey breeds in the Catalonian region. Aranguren-

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Méndez *et al.* (2002) revealed that four breeds with black coats from northern Spain form a closed cluster and supported the hypothesis of a common ancestral past from *Equus asinus europeus*. Ivankovic *et al.* (2002) revealed relatively high levels of heterozygosity in three donkey populations in the coastal region of Croatia. Similarly, high levels of diversity within and between several breeds of Chinese donkey was reported (Zhu and Su 2011, Zhang *et al.* 2016). However, literature on Indian donkey molecular characterization using microsatellite is hard to pin down.

Donkey population of Rajasthan is about 0.081 million (Anonymous 2017b). Small sized light and dark grey donkeys of Rajasthan play an important role in providing draught power in pastoral migration and other draught operations. It is mostly used by nomadic pastoralists in brick kilns for carrying bricks and in pneumatic wheel cart for rural and urban transport. Donkeys are also maintained by people engaged in dairy business to carry the cow dung for disposal (Pal *et al.* 2013). However, it has suffered 20.23% decline between the last two national livestock census (animalhusbandry.rajasthan.gov.in). Considering its adaptation to the harsh environment and need for this area, existing diversity status needs to be established.

The current study aimed to provide insight into the genetic diversity status of local Rajasthan donkey population of India, which will offer a valuable reference for its registration and for designing rational strategies in donkey conservation and breeding programs.

MATERIALS AND METHODS

Breed characteristics and sampling: Rajasthan donkeys are of light brown, dark brown, grey (white) and black colour. However, light brown donkeys are predominant. Belly, inner surfaces of legs, ventral side of neck and inner sides of ears are generally of lighter shade or white in most of the donkeys. The white marking are around muzzle and eyes (Fig. 1). Shoulder strip and dark outline markings are common in light and dark coloured donkeys. The manes is small as compared to horses and is of darker shade than the rest of the body. As per 19th livestock census, donkey population in Barmer, Bikaner and Churu district of Rajasthan was 17,495, 8,712 and 5,063, respectively (animalhusbandry.rajasthan.gov.in). Sufficiently large population provided the scope for selection of unrelated animals. Thus blood samples were collected from 50 randomly selected and unrelated animals from Barmer, Bikaner and Churu districts.

Fig. 1. A representative animal of Rajasthan donkey population.

DNA extraction and quantification: The blood samples were taken from the jugular vein using vacutainer tubes with K2-EDTA (Ethylene diaminetetra acetic acid) anticoagulant. Samples were kept in an ice-box and transported to the laboratory where they were stored at -20°C until used for DNA extractions. Genomic DNA was isolated from blood samples following standard protocol involving lysate of the washed white-cells and phenol-chloroform-isoamylalcohol (25:24:1) extraction (Sambrook *et al.* 1989). After extraction, the quality and quantity of genomic DNA was estimated using Nanodrop spectrophotometer.

Microsatellite polymorphism detection: Microsatellite primer sequences are often conserved across related species and thus can be used for diversity estimation in these species (Seilsuth *et al.* 2016). Therefore, we utilized 20 equine microsatellite markers based on degree of polymorphism and genome coverage (Table 1).

These microsatellites have been shown to be effective markers in the study of genetic diversity of donkeys in previous studies (Jordana *et al.* 2001, Aranguren-Méndez *et al.* 2002, Ivankovic *et al.* 2002, Xie 2004, Zhu and Su 2011). Moreover, these markers adhere to the guidelines of FAO (Anonymous 2017c). Forward primer of each marker was 5' labeled witha fluorescent dye. PCR amplification was performed in a reaction volume of 10 µl (10–20 ng of genomic DNA, 0.2 µM of each primer and DreamTaq Green PCR master mix consisting of 0.2 mM of each dNTP and 2 mM $MgCl_2$). The amplification protocol consisted of initial denaturation of 94°C for 2 min, 30 cycles of 94°C for 45 sec, specific annealing temperature (T_A) for 45 sec, 72°C for 1 min and final extension step at 72°C for 10 min. The amplification products were initially electrophoresed on a 2% agarose gel treated with ethidium bromide for later visualization of DNA bands under ultraviolet light.

Microsatellite marker genotyping: The amplified DNA was multiplexed and genotyped using an automated Genetic Analyzer 3130xl. The genotyping reaction contained 1 µl of PCR products, 8.9 µl of Hi-Di formamide and 0.1 µl of GeneScan-500LIZ size standard. The allele sizing and genotyping results were obtained as a list of alleles using GeneMapper^{TM} software.

Data analyses: The main parameters of genetic variability including allele frequencies, observed (Na) and effective (Ne) number of alleles, observed (Ho) and expected heterozygosity (He) and heterozygote deficit (F_{IS}) in the whole population were calculated by analyzing the individual multilocus genotypes by means of the GenAlEx 6.5 software (Peakall and Smouse 2012). Bottleneck events

Table 1. Characteristics of microsatellites used for diversity estimation in the study

Panel	Microsatellite locus	Horse chromosome	Primer sequence (5' to 3')	Accession number	T_A	Predicted size range* (bp)	Dye
Panel 1	HTG06	15	CCTGCTTGGAGGCTGTGATAAGATGTTCACTGAATGTCAAATTCTGCT	None	58°C	84 – 106	FAM
	HTG04	9	CTATCTCAGTCTTCATTGCAGGACCTCCCTCCCTCCCTCTGTTCTC	None	58°C	127 – 141	FAM
	HMS05**	-	TAGTGTATCCGTCAGAGTTCAAAGCAAGGAAGTCAGACTCCTGGA	None	58°C	-	HEX
	HMS02	10	ACGGTGGCAACTGCCAAGGAAGCTTGCAGTCGAATGTGTATTAAATG	X74631	58°C	218 – 238	HEX
Panel 2	HMS06	4	GAAGCTGCCAGTATTCAACCATTGCTCCATCTTGTGAAGTGTAACTCA	X74635	58°C	153 – 169	HEX
	COR18	25	AGTCTGGCAATATTGAGGATGTAGCAGCTACCCTTTGAATACTG	AF083461	56°C	249 – 271	HEX
	ASB17	2	GAGGGCGGTACCTTTGTACCACCAGTCAGGATCTCCACCG	X93531	58°C	91 – 109	FAM
	COR71	26	CTTGGGCTACAACAGGGAATACTGCTATTTCAAACACTTGGA	AF142608	56°C	190 – 202	HEX
Panel 3	VHL20	30	CAAGTCCTCTTACTTGAAGACTAGAACTCAGGGAGAATCTTCCTCAG	None	58°C	-	FAM
	NVHEQ054	28	AGATGTCCACCTTCTCGCTGCGGGGCTTTTAGGAGGTAACTA	AJ245763	62°C	172 – 186	FAM
	HMS03	9	CCAACTCTTTGTCACATAACAAGACCATCCTCACTTTTTCACTTTGTT	X74632	58°C	150 – 170	HEX
	COR22	22	AAGACGTGATGGGAAATCAAAGAAAGTTTTCAAATGTGCCA	AF101391	56°C	254 – 264	HEX
Panel 4	HTG15***	5	TCTTGATGGCAGAGCCAGGATTTGAATGTCACCATGCGGCACATGACT	None	58°C	128–140	FAM
	COR7	17	GTGTTGGATGAAGCGAATGAGACTTGCCTGGCTTTGAGTC	AF083450	56°C	156 – 170	HEX
Panel 5	AHT05	8	ACGGACACATCCCTGCCTGCGCAGGCTAAGGGGGCTCAGC	None	Touch Down PCR (66-56°C)	130 – 146	HEX
	HTG10	21	CAATTCCCGCCCCACCCCCGGCATTTTTATTCTGATCTGTCACATTT	AF169294	60.5°C	93 – 113	FAM
Panel 6	HTG7	4	CCTGAAGCAGAACATCCCTCCTTGATAAAGTGTCTGGGCAGAGCTGCT	None	55°C	272 – 297	FAM
	AHT04	24	AACCGCCTGAGCAAGGAAGTCCCAGAGAGTTTACCCT	None	58°C	148 – 164	HEX
Panel 7	HMS07	1	CAGGAAACTCATGTTGATACCATCTGTTGTTGAAACATACCTTGACTGT	X74636	57°C	165 – 183	HEX
	VHL209	14	TCTTACATCCTTCCATTACAACTATGATACATATGTACGTGAAAGGAT	Y08451	57°C	84 – 96	FAM

*Details from FAO 2004 (www.fao.org/3/a-aq569e.pdf); **Zhu *et al.* 2013; ***Behl *et al.* 2006.

in the population were tested by the software Bottleneck v1.2.02 (Cornuet and Luikart 1997).

RESULTS AND DISCUSSION

India is an unexplored country in terms of donkey livestock biodiversity. Through the genetic analysis presented here, this study provides the first scientific assessment of the genetic diversity status of Rajasthan donkey popula-tion, which may be of great value for its registration as a breed.

Genetic variability: Twenty equine microsatellite markers were explored for assessing donkey genetic diversity. Microsatellite markers designed for horse worked well with Indian donkey (Bertolini *et al.* 2015). All the loci except HMS7 amplified with the donkey genomic DNA. Allele size ranges observed in this study were comparable with that described in literature for other donkey breeds (Zhang *et al.* 2016). Reasonable polymorphism in Rajasthan Donkey was evident from allelic data based on nineteen microsatellite markers (Table 2).

Overall, 155 different alleles were found. Expected number of alleles varied from 1.043 (ASB17) to 8.79 (AHT05) with the mean of 3.8. On an average, 8.16 alleles per locus were observed. HTG10 presented the highest number (17) of observed alleles per locus. On the other hand, ASB17 and HMS5 loci showed limited number of alleles. Only two alleles were detected for ASB17 and three for HMS5 in this population. HMS5 locus was reported to be polymorphic (PIC= 0.53) in five endangered Spanish donkey breeds (Aranguren-Méndez *et al.* 2002). However, similar to our results, it was less informative being monomorphic in Chinese donkey breeds (Zhang *et al.* 2016). Several studies using cross-species amplification techniques have demonstrated that microsatellite alleles are more variable in natural populations of the species from which they were derived. Studies previously performed with microsatellites derived from cattle (*Bos taurus*) and sheep (*Ovis aries*) reported a difference in variability, with the expected heterozygosity and number of alleles being higher in the species from which the microsatellites were isolated (Hutter *et al.* 1998).

Shannon's information Index (I) is a parameter indicative of the informative degree of a marker. Overall high value of I (1.39±0.15) represents the effectiveness of the microsatellite loci to reveal the genetic variation. Minimum value (I) was observed for ASB17 (0.10) and second lowest for HMS5 (0.51). The low values were in accordance with the less number of alleles recorded for these markers (Table 2). Consequently, both these markers were excluded from subsequent analyses of genetic diversity. All other markers had high I values and thus can potentially be used for diverse genetic applications including linkage mapping, individual identification and parentage testing. Ewens-Watterson test for neutrality revealed that all the selected microsatellite markers were neutral as observed F values lie within the upper and lower limits of 95% confidence region of the expected *F* values. The use of microsatellites with a range of polymorphism reduced the risk of overestimating genetic variability, which might occur with microsatellite exhibiting only high polymorphism. Based on the analysis of the 17 remaining loci, the local donkey population revealed a high level of genetic diversity based on its gene diversity in addition to the average number of alleles per locus. The observed and expected heterozygosity values ranged from 0.023 (COR22) to 1 (AHT4) and from 0.343 (HTG4) to 0.886 (AHT05) with an overall mean of 0.688± 0.06 and 0.675±0.04, respectively (Table 3). Genetic diversity of this magnitude is essential for the long-term survival of the population, because it provides the raw material for adaptation and evolution, especially when environmental conditions change as reported by Fischer *et al.* (2017). That is the reason of recognizing the genetic diversity as a key component of biodiversity.

It is hard to compare the within breed diversity indices of Rajasthan donkey with that of the reported literature. This is due to the fact that scientific reports on microsatellite data in domestic donkeys are obscure even at the global level. The diversity in Rajasthan donkey is higher as compared to the donkeys of Spain and Italy. The mean number of alleles per locus ranged from 7.0 in the Andaluza breed to 7.5 in the Allorquina breed of Spain (Aranguren-Méndez *et al.* 2002). Average expected heterozygosities (He) were reported to be not signicantly different among the five critically endangered Spanish donkey breeds which ranged from 0.637 in the Mallorquina breed to 0.684 in the Zamorano-Leonesa breed. Similarly, in the Amiata donkey

Table 2. Observed (Na) and effective number of alleles (Ne) in Rajasthan donkey and Shannon's information index (I) of selected microsatellites

Locus	N	Na	Ne	I	Allele size range (bp)
HMS2	48	8	5.38	1.80	209-239
HMS5	48	3	1.35	0.51	95-105
HTG4	47	5	1.52	0.69	151-171
HTG6	48	5	2.75	1.18	80-86
ASB17	47	2	1.04	0.10	84
COR18	48	11	5.59	1.97	247-273
COR71	47	9	3.93	1.67	168-204
HMS6	48	6	1.94	0.90	151-167
COR22	44	5	1.62	0.79	246-252
HMS03	48	8	2.87	1.28	132-168
NV54	48	11	6.36	2.05	154-194
VHL20	48	8	3.20	1.38	65-95
COR7	47	7	2.34	1.25	161-171
HTG15	48	5	3.31	1.27	120-134
AHT05	45	14	8.79	2.35	120-152
HTG10	48	17	7.64	2.31	75-125
AHT4	48	11	6.58	2.05	106-154
HTG7	48	13	3.96	1.87	122-154
VHL209	48	7	1.81	0.98	68-92
Mean	47.42	8.16	3.79	1.39	
SE	0.26	0.89	0.53	0.15	

Na, No. of different alleles; Ne, No. of effective alleles, 1/(Sum pi^2); Shannon's information index, -1* Sum (pi * Ln (pi)); where pi is the frequency of the i^{th} allele for the population.

Table 3. Heterozygosity statistics for all loci in Rajasthan donkey

Locus	N	Ho	He	uHe	F_{IS}
HMS2	48	0.771	0.814	0.823	0.053
HTG4	47	0.383	0.343	0.346	-0.118
HTG6	48	0.646	0.636	0.643	-0.015
COR18	48	0.833	0.821	0.83	-0.015
COR71	47	0.787	0.746	0.754	-0.056
HMS6	48	0.479	0.486	0.491	0.013
COR22	44	0.023	0.383	0.387	0.941
HMS03	48	0.604	0.651	0.658	0.072
NV54	48	0.958	0.843	0.852	-0.137
VHL20	48	0.792	0.688	0.695	-0.151
COR7	47	0.553	0.572	0.578	0.032
HTG15	48	0.792	0.698	0.705	-0.134
AHT05	45	0.911	0.886	0.896	-0.028
HTG10	48	0.917	0.869	0.878	-0.055
AHT4	48	1	0.848	0.857	-0.179
HTG7	48	0.792	0.747	0.755	-0.06
VHL209	48	0.458	0.447	0.452	-0.026
Mean	47.41	0.688	0.675	0.682	0.008
SE	0.29	0.06	0.04	0.04	0.06

I (Shannon's information index) =1* Sum (pi * Ln (pi)); Ho (observed heterozygosity) = No. of Hets/N; He (expected heterozygosity)= 1 – Sum pi^2; uHe (unbiased expected heterozygosity)=(2N/(2N–1)) * He; F (fixation index)=(He – Ho)/He = 1 – (Ho/He).

breed of Italy, average number of alleles per locus was moderate (5.61±2.89), ranging from 2 (ASB02) to 13 (HTG7) and mean observed heterozygosity was 0.579 (Ciampolini *et al.* 2007). Mean expected heterozygosity was reported to be 0.609. Genetic variability of Pantesco and two other Sicilian autochthonous donkey breeds (Ragusano and Grigio Siciliano) was also lower than the Rajasthan donkey. Sicilian study included 108 donkeys and a total of only 85 alleles were detected. In comparison, higher allele number (155) was observed in Rajasthan donkey population. The mean number of alleles varied from 3.7 in Pantesco to 5.9 in Ragusano. The three breeds showed a quite low level of gene diversity (He) ranging from 0.471 in Pantesco to 0.589 in Grigio (Bordonaro *et al.* 2012). On the other hand, donkey populations in the Croatian coastal region had the diversity (He=0.64) of similar magnitude (Ivankovic *et al.* 2002). Higher diversity estimates than the current study has only been reported in Chinese donkey. Polymorphisms of 24 microsatellite loci in eight Chinese donkey breeds representing large and medium-sized Chinese donkeys established that the Chinese donkey possesses relatively higher genetic diversity (He=0.80) than donkeys from Spain, Italian and Croatian coastal region (Zhu and Su 2011, Zhang *et al.* 2016).Genetic diversity can be influenced by a range of factors including population size, natural selection, mutation rates, gene flow between populations, introgression from hybridisation and historical effects on these factors such as population bottlenecks (Hobbs *et al.* 2011). The lack of obvious geographic and body size structures in Chinese donkeys implicated the high gene flow. Indeed, the pattern of genetic variation in mtDNA D-loop sequences indicated that both the ass lineages Somali and Nubian have been identified from China. These had undergone population expansion events which has clearly increased the genetic diversity (Lei *et al.* 2007).

Observed heterozygosity (Ho) in Rajasthan donkey was in accordance with the expected heterozygosity of a randomly mating population. Thus departure from Hardy-Weinberg Equilibrium (HWE) and possibility of inbreeding was meager. This was also reflected in the almost nil heterozygote deficits in this population, resulting in a positive F_{IS} value (0.008±0.06), which was not significant ($P \leq 0.05$). Majority (70%) of the loci exhibited heterozygosity excess in the population. The absence of geographic barriers as well as the absence of selection for a specific trait may contribute towards the sufficient existing diversity in the Rajasthan donkey population. Moreover, the strategy of sampling unrelated animals from different villages of multiple districts minimized artificially sub-structuring the sample, the associated Wahlund effect, and the inclusion of related animals. Therefore, the current results are expected to reveal real inbreeding status in the population. It seems that farmers exchange or have exchanged animals between different locations of the state, a fact that has prevented the stratification of genetic diversity. This assumption is supported by the actual performance recording of Rajasthan donkey population undertaken in the field as per technical program of the ICAR-Network Project on Animal Genetic Resources (Annual Report 2016). It has been reported that no scientific breeding or artificial insemination was in practice. Method of donkey breeding was through natural mating and donkey owners even hardly know about the pregnancy status of their donkey mares. Male and female donkeys were left loose in the field when not in use, so controlled breeding is not expected. However, current situation involving small flock size and absence of reproductive strategies may cause undesirable loss of heterozygotes. This trend should be properly monitored by governmental and non-governmental organizations involved in promoting the development of animal husbandry in Rajasthan.

Genetic bottleneck analysis: The results of bottleneck analysis using three tests, viz. Sign test, Standardized differences test and Wilcoxon rank test in each of three models of mutations namely, Infinite allele model (IAM), Two phase model (TPM) and Step wise mutation (SMM) model are summarized in Table 4.

The results revealed that Rajasthan donkey population is not bottlenecked as it has not undergone any recent reduction in population size to the extent that population should deviate from mutation drift equilibrium. SMM, considered to be the most suitable model for microsatellite analysis did show the presence of heterozygote excess under Standardized differences and Wilcoxon rank test. Hence the Mode-shift indicator test was also utilized as a method to detect potential bottleneck. The non-bottleneck populations that are near mutation-drift equilibrium are expected to have a large proportion of alleles with low frequency (Luikart 1997). A graphical representation utilizing allelic class and proportion of alleles showed a normal 'L' shaped distribution (Fig. 3). The L shaped curve indicated the abundance of low frequency (<0.10) alleles. This finding suggested the absence of any detectably large, recent genetic bottleneck (last 40–80 generations) in this population.

The substantial genetic diversity of Rajasthan donkey, as demonstrated by the number of alleles per locus and the heterozygosity suggest that this genetic resource has a high conservation value. In addition, these animals have very good draught power, immunity and stress bearing capabilities (Pal *et al.* 2013). These animals are the only source of livelihood for the lowest strata of the society. However, the lack of recognition as a breed makes it difficult to target for conservation. In fact, the population is not at particular risk of extinction on the basis of its population size (>80,000). Therefore, improved sustainable use of these animals through organized breeding programs merits greater priority than conservation per se.

In summary, molecular data indicate that Rajasthan possesses a very interesting pool of donkey genetic resources with respect to their high genetic diversity. Even though the population size is decreasing, still there is scope of scientific intervention due to high existing diversity as depicted by the average number of alleles per locus, observed heterozygosity, absence of inbreeding as well as bottleneck. Breed registration process for this population must be initiated for management of within-breed diversity and reproduction strategies.

Table 4. Population bottleneck analysis in Rajasthan donkey

Test/Model		IAM	TPM	SMM
Sign test (Number of loci with heterozygosity excess)	Exp	11.08	11.08	11.16
	Obs	10	7	3
	P- value	0.38831	0.04853*	0.00015*
Standardized differences test	T2 value	0.545	-2.729	-8.882
	P- value	0.29275	0.00318*	0.00000*
Wilcoxon rank test (one tail for heterozygosity excess)	P- value	0.25764	0.97993	0.99995

*Rejection of null hypothesis/Bottleneck.

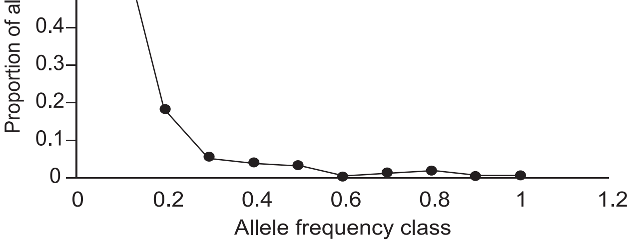

Fig. 2. Graphic representation of proportion of alleles and their distribution in Rajasthan donkey.

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