



Genetic characterization of brown type donkeys of Andhra Pradesh using microsatellite markers

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ABSTRACT

The brown type donkeys of Andhra Pradesh, which are mainly concentrated in Kurnool and Anathapur districts, were evaluated for within breed genetic diversity and bottlenecks using heterologous microsatellite markers. The genomic DNA, isolated from 28 blood samples collected from Kurnool district, were amplified by PCR using FAM and HEX labeled primers and resolved for alleles on automatic DNA sequencer. In all, twenty loci of the horse origin were tested and only 12 loci gave scorable results. Rest of the loci either did not amplify (HMS3 and HMS7) or did not resolve properly (VHL20) or showed less than 4 alleles (HMS5, HMS6, HTG4, ASB17 and COR22) in the studied population. At the 12 loci included in the final analysis, the PCR product size range varied from 76–92 bp at locus HTG6 to 257–273 bp at locus COR18. The observed number of alleles varied from 4 (VHL209) to 10 (AHT5 and HTG7) with a mean of 6.92 ± 1.83 . The effective number of alleles ranged from 1.62 (VHL209) to 7.91 (AHT5) with a mean of 4.21 ± 2.06 . The observed heterozygosity ranged from 0.32 (HMS2) to 0.92 (AHT5) with a mean of 0.57 ± 0.2 . The expected heterozygosity ranged between 0.39 (VHL209) to 0.89 (AHT5 and HTG7) with a mean of 0.72 ± 0.14 . The mean genetic diversity estimate (F_{IS}) was 0.21 indicating moderately high levels of inbreeding. The cumulative exclusion probability (PE) of these loci was 0.999892 indicating their suitability for parentage testing in these donkeys. The sign test, standardized differences test, the Wilcoxon test using the allelic frequency data at the studied loci as well as normal 'L' shaped distribution of the allelic frequency indicated the absence of any recent genetic bottleneck in Brown type donkeys of Andhra Pradesh. When these donkeys were compared to Spiti donkeys of Himachal Pradesh on the basis of allelic frequency data at these loci, they showed Nei's standard and unbiased genetic distances of 0.32 and 0.29, respectively.

Key words: Andhra Pradesh, Diversity, Donkey, Microsatellites

According to livestock census, the total population of donkeys in Andhra Pradesh is 10,517 which comprises 5,196 males and 5,321 females (Census 2012). Majority of the donkeys in Andhra Pradesh are predominantly of the light brown to brown coat colour with lighter bellies. The mean heights at wither of brown type animals are 94.57 ± 5.24 and 89.82 ± 3.36 cm in adult males and females, respectively (Behl *et al.* 2016). The donkey population in Andhra Pradesh, including the brown type donkeys, has shown a steep decline of 74% as compared to their population in 2007 and requires immediate attention and conservation measures. In breed conservation or improvement programme, genetic evaluation of the concerned breed is a major pre-requisite. The microsatellite markers, which are highly polymorphic, dispersed throughout the eukaryotic genome, follow codominant inheritance, can be easily amplified using polymerase chain reaction and resolved

easily for alleles on automated DNA sequencer, are the markers of choice for evaluation of the genetic diversity of a population (Takezaki and Nei 1996, Goldstein and Shlotterer 1999). Increasing number of donkey breeds have been now evaluated for genetic diversity using heterologous microsatellite markers of horse origin (Aranguren-Mendez *et al.* 2001, Jordana *et al.* 2001, Ivankovic *et al.* 2002, Kruger *et al.* 2005, Zhao *et al.* 2005, Bordonaro *et al.* 2012, Colli *et al.* 2013). The present study was undertaken to evaluate brown type donkeys of Andhra Pradesh for genetic diversity and to understand whether they have experienced any recent genetic bottlenecks using microsatellite markers.

MATERIALS AND METHODS

Blood samples (28) were collected from the Kurnool district of Andhra Pradesh in EDTA coated vacutainers. Genomic DNA was isolated by standard procedure of digestion with proteinase K, extraction with phenol/chloroform and precipitation with ethanol. The isolated genomic DNA samples were stored at -20°C and working dilutions were stored at 4°C .

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Table 1. Annealing temperature (°C), PCR product size range (bp), observed (N_O) and effective (N_e) number of alleles, observed (H_O) and expected heterozygosity (H_e), polymorphism information content (PIC), probability of exclusion (PE) and F_{IS} at 12 heterologous microsatellite loci in Brown type donkeys of Andhra Pradesh

Locus	Annealing temperature (°C)	Size-range (bp)	N_O	N_e	H_O	H_e	PIC	PE	F_{IS} [#]
HTG6	58	76–92	6	2.51	0.56	0.61	0.60	0.330	0.09
HTG15	58	118–136	5	3.15	0.65	0.70	0.67	0.413	0.07
AHT5*	59	126–152	10	7.91	0.92	0.89	0.87	0.744	–0.03
HTG7*	59	136–156	10	7.68	0.36	0.89	0.87	0.783	0.60 [#]
HTG10	58	91–103	7	4.03	0.67	0.77	0.73	0.530	0.13
HMS2*	58	229–243	7	5.0	0.32	0.82	0.80	0.606	0.61 [#]
AHT4	58	136–158	8	5.59	0.78	0.84	0.82	0.651	0.08
NVHEQ54	61	164–190	6	2.53	0.36	0.62	0.59	0.366	0.42 [#]
COR18	56	257–273	6	4.97	0.78	0.82	0.79	0.599	0.04
VHL209	55	82–88	4	1.62	0.35	0.39	0.37	0.214	0.11
COR7	59	160–174	8	2.73	0.60	0.65	0.62	0.436	0.08
COR71*	59	172–204	6	2.78	0.50	0.68	0.62	0.435	0.26
Mean			6.92±1.83	4.21±2.06	0.57±0.2	0.72±0.14	0.69±0.15	0.999892 [§]	0.21

*Locus deviated significantly from Hardy Weinberg equilibrium ($P < 0.05$); [§]Cumulative PE; [#]Significant heterozygote deficit ($P < 0.05$).

The genomic DNA was amplified by PCR using heterologous microsatellite markers of horse origin. Each 25 μ l reaction consisted of DNA (about 100 ng), primers (7.5 pmol each), dNTPs (200 μ M each), 10 \times buffer (50 mM KCL, 10 mM tris-HCl, 0.1% gelatin), MgCl₂ (1.5 mM) and Taq DNA polymerase (1 unit). The thermocycling conditions included an initial denaturation at 94°C for 2 min followed by 30 cycles of 45 sec at 92°C, 45 sec at annealing temperature (Table 1) and 1 min at 72°C. A final extension step was carried at 72°C for 15 min. The primers were labeled with HEX and FAM to facilitate resolution of alleles on automated DNA sequencer.

The within-breed genetic diversity parameters of observed and effective number of alleles, observed and expected heterozygosity at each locus were calculated using POPGENE computer program version 1.31 (Yeh *et al.* 1999). The polymorphism information content (PIC) at each locus was calculated according to Botstein *et al.* (1980). The probability of parentage exclusion (P) at each locus was determined as described by Jamieson and Taylor (1997). The cumulative probability of parentage exclusion (PE) was calculated as

$$PE = 1 - (1 - P_1)(1 - P_2)(1 - P_3) \dots (1 - P_k)$$

The exact test for Hardy-Weinberg equilibrium at each locus and linkage disequilibrium between pairs of loci was calculated using GENEPOP software (Raymond and Rousset 1995, Rousset 2008) after applying 100 batches of 1000 iterations to calculate the exact probabilities. The within-breed gene diversity (F_{IS}) and its level of significance was computed as described by Weir and Cockerham (1984) after 240 randomizations of the data using FSTAT computer program, version 2.3.9 (Goudet 2001). The data was also analysed for any recent genetic bottlenecks using BOTTLENECK computer program (Piry *et al.* 1999). Nei's

standard (Nei 1972) and unbiased (Nei 1978) genetic distances were calculated by POPGENE computer program (Yeh *et al.* 1999).

RESULTS AND DISCUSSION

The twelve loci used in the final analysis, amplified well, produced unambiguous allele patterns and showed more than four alleles. In all, twenty loci were tested. The loci that either did not amplify well (HMS3 and HMS7) or did not resolve properly (VHL20) or showed less than four alleles (HMS5, HMS6, HTG4, ASB17 AND COR22), were not included in the final analysis.

At the twelve loci included in the final analysis, the PCR product size ranged from 76–92 bp at locus HTG6 to 257–273 bp at locus COR18 (Table 1). The observed number of alleles varied from 4 (VHL209) to 10 (AHT5 and HTG7) with a mean of 6.92±1.83. The effective number of alleles ranged from 1.62 (VHL209) to 7.91 (AHT5) with a mean of 4.21±2.06. The observed heterozygosity ranged from 0.32 (HMS2) to 0.92 (AHT5) with a mean of 0.72±0.14.

The mean PIC for all loci assessed from allele frequency data was 0.71±0.15, ranging from 0.37 (VHL209) to 0.78 (HTG7) indicating their suitability for genetic variability studies in Indian donkey breeds (Table 1). The cumulative PE of 0.999892 at these twelve loci indicated the suitability of this set of 12 loci for parent exclusion studies in these donkeys.

In exact test for Hardy-Weinberg disequilibrium at each locus, only four loci (AHT5, HTG10, HMS2 and COR71) deviated significantly ($P < 0.05$) from Hardy-Weinberg equilibrium. All possible pairs of loci were also tested for linkage disequilibrium with only 7 pairs out of 66 pairs showed significant ($P < 0.05$) linkage. However, nothing can be said conclusively about these 7 pairs because loci being heterologous their chromosomal locations were not known.

The F_{IS} estimates for individual loci revealed that only one loci showed heterozygosity excess (AHT5) which was not significant. The rest of the loci showed heterozygote deficit with three of the loci (HTG7, HMS2 and NVHEQ54) showing significant ($P < 0.05$) heterozygote deficit (Table 1). The overall F_{IS} values of 0.21 were significant at $P < 0.05$. The likely reason for 21% heterozygote deficiency seems to be mating of these donkeys restricted mostly to the small population available within the village, presence of null alleles (non-detectable alleles) or presence of population substructure leading to Wahlund effect (Nei 1987).

These donkeys were also evaluated for any recent genetic bottlenecks. In the sign test which tests the probability of getting significant number of loci with heterozygote excess, the probability values of 0.57, 0.59 and 0.16 under infinite allele model (IAM), two phase mutation model (TPM) and step-wise mutation model (SMM), respectively, indicated absence of any recent genetic bottleneck in Brown type donkeys of Andhra Pradesh (Cornuet and Luikart 1996). In standardized differences test, T_2 values of 1.51, -0.024 and -2.618 under IAM, TPM and SMM also indicated absence of any recent genetic bottleneck in Brown type donkeys of Andhra Pradesh. The Wilcoxon test also showed absence of any recent genetic bottlenecks with probability values of 0.129, 0.91 and 0.301 for combined heterozygosity excess and deficit under IAM, TPM and SMM. Same results were reflected by normal 'L' shaped (Fig. 1) allelic frequency distribution suggesting absence of any recent genetic bottleneck in Brown type donkeys of Andhra Pradesh. Though, the population of Brown type donkeys of Andhra Pradesh along with overall population of donkeys has decreased drastically in recent times and mating of these donkeys within the animals available in the village has led to the overall heterozygote deficiency of 21% in these donkeys, luckily, as indicated by the results of above tests, no genetic bottlenecks were detected in Brown type donkeys of Andhra Pradesh. The genetic bottlenecks in a population are generally detected when the population size is reduced to 20–30 breeding animals only (Luikart *et al.* 1998).

Based on the allele frequency data at eleven common loci, these donkeys were also compared with Spiti donkeys of Himachal Pradesh. The important genetic diversity

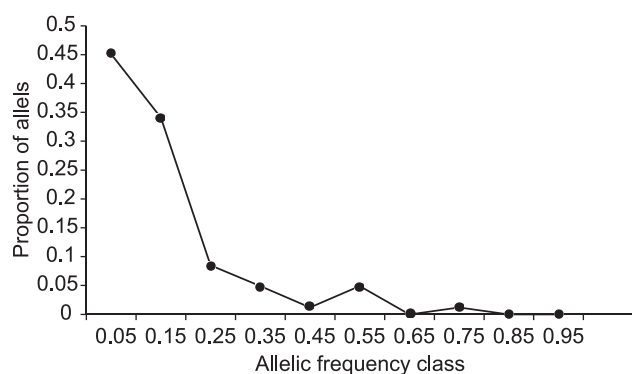


Fig. 1. Normal 'L' shaped plot of allelic frequency class with proportion of alleles showing absence of any recent genetic bottlenecks in Brown type donkeys of Andhra Pradesh.

Table 2. PCR product size range (bp), observed (N_o) and effective (N_e) number of alleles, observed and expected heterozygosity, at studied heterologous microsatellite loci in Spiti donkeys of Himachal Pradesh

Locus	Allele size range (bp)	N_o	N_e	H_o	H_e
HTG6	183–201	4	2.93	0.78	0.67
HTG15	78–87	5	3.49	0.80	0.72
HTG7	225–239	10	4.84	0.89	0.80
HTG10	78–90	7	3.39	0.87	0.71
HMS2	84–103	8	2.89	0.65	0.66
AHT4	132–157	8	4.74	0.78	0.80
NVHEQ54	132–144	6	2.30	0.48	0.57
COR18	79–85	6	4.45	0.72	0.78
VHL209	189–203	6	2.03	0.52	0.51
COR07	161–172	6	2.63	0.65	0.63
COR71	253–271	6	3.74	0.80	0.74
Mean±SD		6.33 ±1.72	3.35 ±0.94	0.72 ±0.13	0.69 ±0.09

parameters at these eleven loci for Spiti donkeys are given in Table 2. The Nei's standard and unbiased distances of 0.32 and 0.30 indicated genetic distinctness of these two breeds which was in agreement with the geographical distances between the distribution areas of these breeds.

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