ONLINE RESOURCES

Low genetic diversity of the endangered Indian wild ass *Equus hemionus khur,* as revealed by microsatellite analyses

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Running title: Genetic diversity of the endangered Indian wild ass

Keywords: Indian wild ass, Equus, khur, microsatellite, bottleneck.

Similar to other Asiatic wild asses, the Indian wild ass (*Equus hemionus khur)* belongs to an endangered wild species/subspecies of wild asses, which were once widely distributed from the Arabian Peninsula to Manchuria before being confined to fragmented, isolated, dry, and harsh habitats in Asia (www.iucnredlist.org). Two major species of the Asiatic wild asses, *Equus hemionus* and *Equus kiang,* have been described on the basis of morphology and karyotyping (Groves *et al.* 1967; Ryder and Chemnick 1990). In addition, five subspecies of *E. hemionus* (*E. h. hemionus*, *E. h. onager*, *E. h. kulan*, *E. h. luteus*, and *E. h. khur)* have been identified (Schaller 1998; Shah 2002); however, recently emerged molecular information failed to justify the variation in the subspecies (Vilstrup *et al.* 2013; Rosenbom *et al.* 2015; Khaire *et al.* 2016). Previously, *E. h. khur* was distributed from southern Pakistan (Sindh and Baluchistan provinces) and Afghanistan to southeastern Iran. However, currently, the Indian wild ass sanctuary (4900km^2), located in the Little and Great Rann of Kutch in Gujarat, is the ultimate refugia of the Asiatic wild ass population in southern Asia. In this pilot study, we genotyped 42 free-ranging Indian wild asses at five microsatellite loci to reveal the genetic diversity at a nuclear level. Faecal samples of the free-ranging Indian wild asses (*E. h. khur)* were collected from three localities in the Indian wild ass sanctuary in the Little Rann of Kutch (Fig 1). Permission for collecting the faecal samples in the Indian wild ass sanctuary was granted by the Principal Chief Conservator of Forest (Wildlife), Gujarat (Letter No. WLP/28/C/574–76/2013–14; Date: 18/12/2013). Sample collection and genomic DNA extraction procedures are described in our previous study on Indian wild ass (Khaire *et*

al. 2016). Five microsatellite loci (LEX74, COR58, ATH5, COR112, and HMS6),were used (Table 1). The forward primer of each locus was fluorescent labelled at the 5′ end. PCR was performed in 20 μL of reaction mixture, which comprised 3 μL of deionised water, 8 μL of DreamTaq Master Mix (Thermo Scientific, Waltham, MA), 1.5 μL of 2.5 mM MgCl2, 1 μL of 0.3% bovine serum albumin, 0.3 μL (5 U/μL) Platinum® Taq DNA Polymerase (Invitrogen™), 0.8 μL of each primer, and variable amounts of genomic DNA. PCR cycling for all the five microsatellite loci was performed using a PCR Veriti 96-well thermal cycler (Applied Biosystems, Waltham, MA). The PCR mixture was subjected to initial denaturation at 94 °C for 15 min, followed by 45 cycles at 94 °C for 50 s, 50 s for annealing of primers (annealing temperature varied according to the microsatellite locus, Table 1), and amplicons were extended at 72 °C for 45 s followed by a final extension at 72 °C for 20 min. After staining with GelRed™, amplified fragments were visualised on 2% agarose gel. Depending on the quality of amplification, the samples were diluted in water, mixed with formamide and LIZ® 500-bp internal size standard (Applied Biosystems™), and loaded into a 3500 Genetic Analyzer® (Applied Biosystems™) sequencer. GeneMapper® v4.1 (Applied Biosystems™) was used to visualise the results. Reliability of the genotype data obtained through the non-invasive method was confirmed using a multiple tube approach described by Taberlet *et al.* (1996). Deviation from the Hardy–Weinberg equilibrium, gametic disequilibrium, null allele frequencies, and *F*is values (Weir and Cockerham 1984) for each pair were calculated using GENEPOP software v4.5.1 (Raymond and Rousette 1995). Using the same programme, *P* values were estimated from the Markov chain algorithm by using 10,000 dememorization steps, 500 batches, and 5000 iterations per batch. Number of alleles (Na), observed heterozygosity (Ho) and expected heterozygosity (He), and polymorphic information content (PIC) were calculated using Cervus software v3.0 (Kalinowski *et al.* 2007). Occurrence of null-alleles and scoring errors were confirmed using MICROCHECKER 2.2.3 software (Van Oosterhout *et al.* 2004). Four of the five loci tested in 42 Indian wild asses were polymorphic. One locus, HMS6, was inconsistent in amplification or monomorphic; hence, it was not analysed further. The Na ranged from 4 (ATH5) to 7 (LEX74) and He ranged from 0.147 (ATH5) to 0.686 (LEX74) (Table 2). The mean Na across loci was 5.50. Mean He and mean PIC were 0.474 and 0.435, respectively, indicating a bottleneck (Table 2). The low mean heterozygosity of the free-ranging Indian wild ass population is corroborated by the 1967 Gujarat forest reports. According to these reports, the present population of approximately 4000 Indian wild asses in the sanctuary expanded from the 362 individuals surviving a dreadful protozoic disease, Surra, in 1962 in Gujarat. Our previous study of Indian wild asses from the same region, which used mitochondrial DNA markers,

also supports this finding (Khaire *et al.* 2016). Combining all, we infer that the reduced genetic diversity found in the present ~4000 free-ranging Indian wild asses inhabiting the sanctuary is the outcome of bottleneck inflicted on this population by the protozoan disease Surra in the year 1962 (Hoelzel *et al.* 2002).No linkage disequilibrium was detected among all possible loci pairs. Except the COR112 locus, all three loci highly deviated from the Hardy–Weinberg proportion ($P = 0.001$). Moreover, except the COR112 locus, highly significant heterozygosity deficit ($P < 0.001$) levels resulting due to non-random mating was detected in the remaining loci. The COR112 locus linked to the major histocompatibility complex of the immune system yielded 6 alleles and resulted in 0.343 and 0.322 of He and PIC, respectively (Table 2). Two of the four loci exhibited moderately high estimated frequency of null alleles, and among them, COR112 was the only locus to exhibit a high estimated frequency of null alleles (Table 2). MICROCHECKER 2.2.3 output revealed no evidence of large allele drop out and stutter peaks, but show possibility of null allele on three loci, except, locus LEX74 due to general excess of homozygotes for most allele size classes over these loci. High values for null alleles were observed owing to PCR failure, which most probably occurred because of mutations in the annealing sites or inconsistent DNA template quality, which caused some loci to amplify easily, whereas others failed (Dakin and Avise 2004). In our study, we did not exclude any of these causes because all our samples were faecal samples, which often yield low quantity/quality DNA extracts (Taberlet *et al.* 1996). To resume, this pilot study was the first attempt to assess the genetic diversity of Indian wild ass by using published *Equus* microsatellite markers to provide a brief report on the population genetics of free-ranging endangered Indian wild asses in the Indian wild ass sanctuary. In the future, genotyping more microsatellite loci along with a genome-wide association study would contribute to the conservation of this sole surviving population of Asiatic wild asses in southern Asia.

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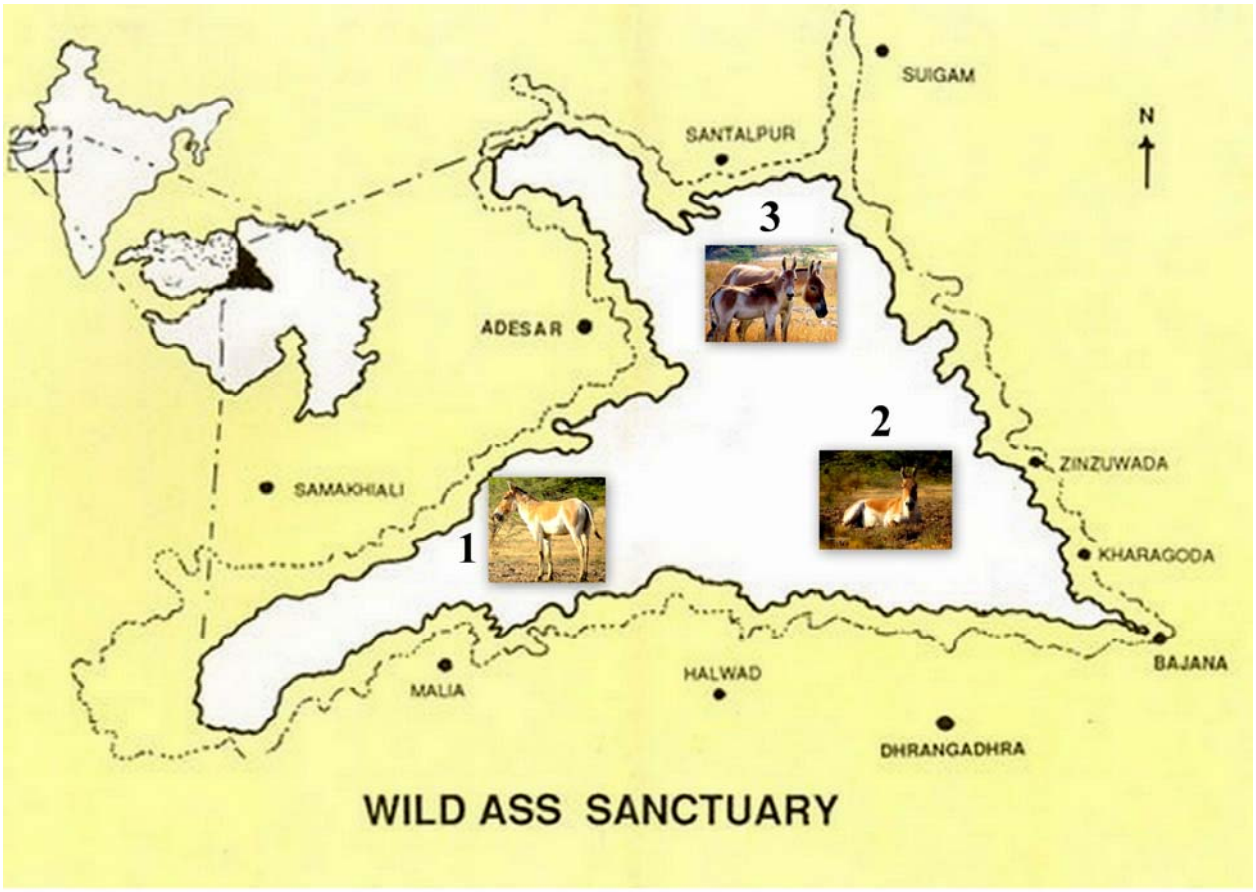
Received 7 July 2016, in revised form 11 September 2016; accepted 23 September 2016

Unedited version published online: 26 September 2016

Figure Legend

Figure 1.

The Indian ass sanctuary map showing sampling location. Number of sampled individuals are indicated in parenthesis. Map modified from Indian wild ass sanctuary site map at https://forests.gujarat.gov.in/wild-ass-sanctuary.htm. Photographs of Indian wild ass are the original field photographs captured during sampling



1: Jogad (15)

2 :Bajana (12)

3 :Zinzuwada (15)

Table 1. Details of five microsatellite loci used for genotyping Indian wild ass, *Equus hemionus khur*

Locus	Primer Sequence(5'-3')	Primer Dye	Allele Size	TA (^{0}C)	Reference
LEX74	5'- AAG AGT GCT CCC GTG TG-3' 5'-GAC AAT GCA GAA CTG GGT AA-3'	JOE	184- 192 bp	55.6	Bailey *et al.* 2000
COR58	5'-GGG AAG GAC GAT GAG TGA-3' 5'-CAC CAG GCT AAG TAG CCA AAG-3'	6FAM	220-234 bp	60.4	Ruth *et al.*1999
AHT5	5'-ACG GAC ACA TCC CTG CCT GC-3' 5'-GCA GGC TAA GGG GGC TCA GC-3'	HEX	126-176 bp	65.6	Binns *et al.*1995
COR112	5'-TTA CCT GGT TAT TGG TTA TTT GG-3' 5'-TCA CCC ACT AAA TCT CAA ATC C-3'	TAMRA	251- 271 bp	57.9	Tseng *et al.* 2010
HMS6	5'-GAAGCTGCCAGTATTCAACCATTG-3' 5'-CTCCATCTTGTGAAGTGTAACTCA-3'	HEX	176-188 bp	62.6	Guerin *et al.*1994

Table 2. Summary statistics of Indian wild ass, *Equus hemionus khur* (N=42)

Locus	*n*	*Na*	*He*	*Ho*	PIC	*F* (null)	*Fis*
LEX74	37	7	0.686	0.718	0.659	0.195	0.046**
COR58	32	5	0.690	0.143	0.620	0.450	0.799**
ATH5	39	4	0.147	0.026	0.140	0.186	0.827**
COR112	35	6	0.343	0.250	0.322	0.810	0.277*

n=sample size per locus , *Na* =number of alleles, H_E = expected heterozygosity, $H_{O=}$ observed heterozygosity, PIC= polymorphic information content ,*F* (null)= estimated null allele frequency, *Fis* =(Weir and Cockerham 1984), **P* <0.01, ***P*<0.001