ONLINE RESOURCES

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

DEVENDRA KHAIRE, ASHWIN ATKULWAR, SAMEERA FARAH and MUMTAZ BAIG*

Laboratory of Molecular and Conservation Genetics, Department of Zoology, Govt. Vidarbha Institute of Science and Humanities, Amravati 444 604, India

[Khaire D., Atkulwar A., Farah S. and Baig M. 2017 Low genetic diversity of the endangered Indian wild ass *Equus hemionus khur*, as revealed by microsatellite analyses. *J. Genet.* **96**, e31–e34. Online only: http://www.ias.ac.in/jgenet/OnlineResources/96/e31.pdf]

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, *E. hemionus* and *E. kiang*, have been described on the basis of morphology and karyotyping (Groves and Mazak 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 southeast Iran. However, currently, the Indian wild ass sanctuary (4900 km^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 (figure 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; dated: 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 denoised water, 8 μL of DreamTaq Master Mix (Thermo Scientific, Waltham, USA), 1.5 μL of 2.5 mM $MgCl_2$, 1 μL of 0.3% bovine serum albumin, 0.3 μL (5 U/μL) Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, USA), 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, USA). 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 visualized 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® ver. 4.1 (Applied Biosystems) was used to visualize the results. Reliability of the genotype data obtained through the noninvasive 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 ver. 4.5.1 (Raymond

*For correspondence. E-mail: mumtazbaig@gmail.com.

Keywords. Indian wild ass; microsatellite; bottleneck; *Equus hemionus khur*.

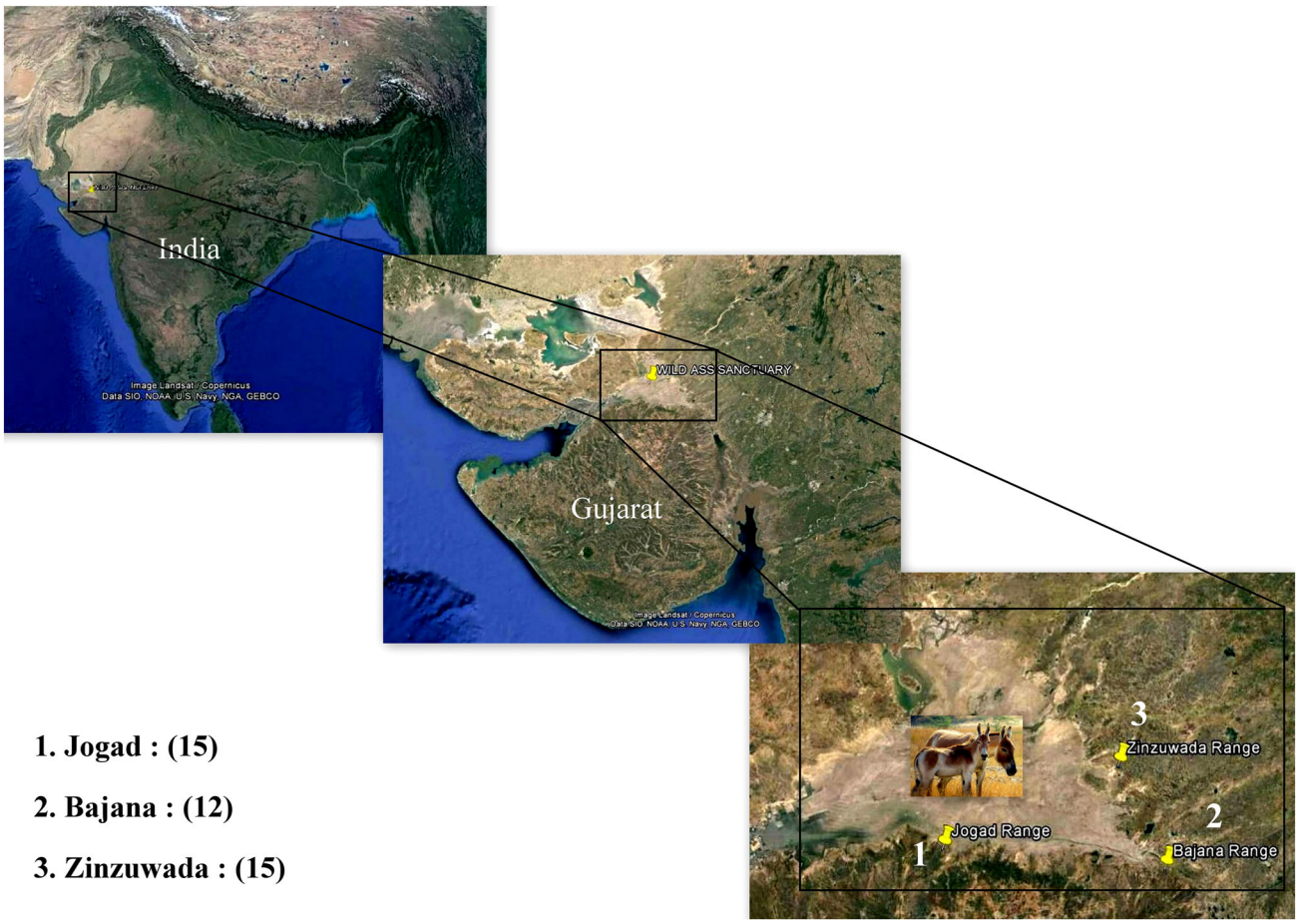

Figure 1. The Indian ass sanctuary map showing sampling location. Number of sampled individuals are indicated in parenthesis. Map has taken and modified from Google Earth. Photograph of Indian wild ass is the original field photographs captured during sampling.

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

Locus	Primer sequence (5′–3′)	Primer dye	Allele size (bp)	T_a (°C)	References
LEX74	5′-AAG AGT GCT CCC GTG TG-3′ 5′-GAC AAT GCA GAA CTG GGT AA-3′	JOE	184–192	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	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	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	57.9	Tseng *et al.* (2010)
HMS6	5′-GAAGCTGCCAGTATTCAACCATTG-3′ 5′-CTCCATCTTGTGAAGTGTAACTCA-3′	HEX	176–188	62.6	Guérin *et al.* (1994)

and Rousset 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 (N_a), observed heterozygosity (H_o) and expected heterozygosity (H_e), and polymorphic information content (PIC) were calculated using Cervus software ver. 3.0 (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 N_a ranged from 4 (ATH5) to 7 (LEX74) and H_e ranged from 0.147 (ATH5) to 0.686 (LEX74) (table 2). The mean N_a across loci was 5.50. Mean H_e and mean PIC were 0.474 and 0.435, respectively, indi-

Table 2. Summary statistics of Indian wild ass, *E. h. khur* ($n = 42$).

Locus	n	N_a	H_e	H_o	PIC	F (null)	F_{IS}
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; N_a, number of alleles; H_e, expected heterozygosity; H_o, observed heterozygosity; PIC, polymorphic information content; F (null), estimated null allele frequency; F_{IS}, Weir and Cockerham 1984; * $P < 0.01$; ** $P < 0.001$.

cating 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 ~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 nonrandom mating was detected in the remaining loci. The COR112 locus linked to the major histocompatibility complex of the immune system yielded six alleles and resulted in 0.343 and 0.322 of H_e 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 occurred most probably 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 genomewide association study would contribute to the conservation of this sole surviving population of Asiatic wild asses in southern Asia.

References

Bailey E., Skow L., Bernoco D., DelValle A., Scavone M. D. P., Bowling A. T. and Murray J. D. 2000 Equine dinucleotide repeat loci LEX071 through LEX078. *Anim. Genet.* **3**, 286–287.

Binns M. M., Holmes N. G., Rolliman A. and Scott A. M. 1995 The identification of polymorphic microsatellite loci in the horse and their use in thoroughbred parentage testing. *Br. Vet. J.* **151**, 9–15.

Dakin E. E. and Avise J. C. 2004 Microsatellite null alleles in parentage analysis. *Heredity* **93**, 504–509.

Groves C. P. and Mazak V. 1967 On some taxonomic problems of Asiatic wild asses; with the description of a new subspecies (Perissodactyla; Equidae). *Z. Saugetierkd.* **32**, 321–355.

Guérin G., Bertaud M. and Amigues Y. 1994 Characterization of seven new horse microsatellites: HMS1, HMS2, HMS3, HMS5, HMS6, HMS7 and HMS8. *Anim. Genet.* **25**, 62.

Hoelzel A. R., Fleischer R. C., Campagna C., Le Boeuf B. J. and Alvord G. 2002 Impact of a population bottleneck on symmetry and genetic diversity in the northern elephant seal. *J. Evol. Biol.* **15**, 567–575.

Kalinowski S. T., Taper M. L. and Marshall T. C. 2007 Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol. Ecol.* **16**, 1099–1106.

Khaire D., Atkulwar A., Farah S. and Baig M. 2016 Mitochondrial DNA analyses revealed low genetic diversity in the endangered Indian wild ass *Equus hemionus khur. Mitochondrial DNA Part A* **12**, 1–6.

Raymond M. and Rousset F. 1995 GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J. Hered.* **86**, 248–249.

Rosenbom S., Costa V., Chen S., Khalatbari L., Yusefi G., Abdukadir A. *et al.* 2015 Reassessing the evolutionary history of ass-like equids: insight from pattern of genetic variation in contemporary extant populations. *Mol. Phylogenet. E.* **85**, 88–96.

Ruth L. S., Hopman T. J., Schug M. D., Aquadro C. F., Bowling A. T., Murray J. D. *et al.* 1999 Equine dinucleotide repeat loci COR041-COR060. *Anim. Genet.* **30**, 320–321.

Ryder O. A. and Chemnick L. G. 1990 Chromosomal and molecular evolution in Asiatic wild asses. *Genetica* **83**, 67–72.

Schaller G. B. 1998 *Wild life of the Tibetan Steppe.* University of Chicago Press, Chicago, USA.

Shah N. 2002 Status and action plan for the Kiang (Equuskiang). In *Status survey and conservation action plan equids: zebras, asses and horses, gland* (ed. P. D. Moehlman). IUCN, Switzerland.

Taberlet P., Griffin S., Goossens B., Questiau S., Manceau V., Escaravage N. *et al.* 1996 Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Res.* **24**, 3189–3194.

Tseng C. T., Miller D., Cassano J., Bailey E. and Antczak D. F. 2010 Identification of Equine major histocompatibility

complex haplotypes using polymorphic microsatellites. *Anim. Genet*. **41**, 150–153.

Van Oosterhout C., Hutchinson F., Wills M. and Shipley P 2004 Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* **4**, 535–538.

Vilstrup J. T., Seguin-Orlando A., Stiller M., Ginolhac A., Raghavan M. and Nielsen S. C. 2013 Mitochondrial phylogenomics of modern and ancient equids. *PLoS One* **8**, e55950.

Weir B. S. and Cockerham C. 1984 Estimating F-statistics for the analysis of population structure. *Evolution* **38**, 1358–1370.

Received 7 July 2016, in final revised form 11 September 2016; accepted 23 September 2016
Unedited version published online: 26 September 2016
Final version published online: 27 June 2017

Corresponding editor: INDRAJT NANDA