Mitochondrial DNA analyses revealed low genetic diversity in the endangered Indian wild ass Equus hemionus khur

Devendra Khaire, Ashwin Atkulwar, Sameera Farah and Mumtaz Baig

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

ABSTRACT
The Indian wild ass Equus hemionus khur, belonging to ass-like equid branch, inhabits the dry and arid desert of the Little Rann of Kutch, Gujarat. The E. h. khur is the sole survivor of Asiatic wild ass species/subspecies in South Asia. To provide first ever insights into the genetic diversity, phylogeny, and demography of the endangered Indian wild ass, we sampled 52 free-ranging individuals from the Little Rann of Kutch by using an non-invasive methodology. The sequencing of 230 bp in cytochrome b (Cyt b) and displacement loop (D-loop) region revealed that current ~4000 extant population of Indian wild ass harbours low genetic diversity. Phylogenetic analyses confirmed that E. h. khur, E. h. onager, and E. h. kulan belong to a single strict monophyletic clade. Therefore, we suggest the delimitation of the five E. hemionus subspecies in vogue to a single species E. hemionus. The application of molecular clock confirmed that the Asiatic wild ass had undergone diversification 0.65 Million years ago. Demographic measurements assessed using a Bayesian skyline plot demonstrated decline in the maternally effective population size of the Indian wild ass during different periods; these periods coincided with the origin and rise of the Indus civilization in the north west of the Indian subcontinent during the Neolithic. In conclusion, maintaining high genetic diversity in the existing isolated population of 4000 Indian wild ass inhabiting the wild ass sanctuary is important compared with subspecies preservation alone.

Introduction
Equids, belonging to the family Equidae, include asses, zebras, extant horses, and extinct horse-like species. Despite their vast fossil records and them being a classic example of macroevolution over many years, members of Equidae remain debatable amongst researchers (MacFadden 2005). Currently, IUCN has recognized eight extant species in the genus Equus: Horse (Equus caballus), Przewalski horse (E. przewalskii), African wild ass (E. africanaus), kiang (E. kiang), Asiatic wild ass (E. hemionus), plains zebra (E. quagga), mountain zebra (E. zebra), and Grevy’s zebra (E. grevyi) (Moehlman 2002). Because of its rapid radiation and recent divergence, the genus Equus has been extensively studied for addressing questions mainly related to its phylogeny. Most previous studies on equids were based on few samples obtained mainly from captive individuals from zoos and other sources having limited information regarding their exact geographical origin (Oakenfull et al. 2000; Steiner & Ryder 2011; Steiner et al. 2012). Among all equid branches, the ass-like branch of equids comprising of the Asiatic and African wild ass remains largely unresolved and requires attention. Rapid climate change and increased anthropogenic pressure on their habitats have fragmented the current distribution of all Asiatic wild asses. On the basis of their morphology and karyotype, two main species of the Asiatic wild ass, E. hemionus and E. kiang, have been described (Groves & Mazák 1967; Ryder & Chemnick 1990); however, molecular data failed to distinguish these species (McCue et al. 2008; Vilstrup et al. 2013). Nonetheless, three to five debatable subspecies of E. hemionus and E. kiang have been geographically described for each species. At present, five subspecies of E. hemionus, E. h. onager, E. h. kulan, E. h. luteus, and E. h. khur, have been identified (Groves & Mazák 1967; Schaller 1998; Shah 2002). Similarly, three subspecies of E. kiang, E. k. kiang, E. k. holdereri, and E. k. polygonon, have been recognized (Shah 2002; Grubb 2005). This study focuses on one such Asiatic wild ass population from western India called E. h. khur. The E. h. khur inhabits the highly fragmented, dry, and arid desert of the Little Rann of Kutch, Gujarat, India (Figure 1). Moreover, it is the sole survivor of the once widespread Asiatic wild ass population, showing continuous distribution from the Arabian peninsula to Manchuria, and declared endangered by the IUCN (Grubb 2005). Equus hemionus khur is the flagship species of the coastal desert in western India, a unique ecosystem spread into an area of 9000 km², encompassing the Great and Little Rann of Kutch (22°–25° N, 68°–71° E). In the past, E. h. khur was distributed from southern Pakistan (Sindh and Baluchistan provinces) and Afghanistan to southeastern Iran. However, the Indian wild ass sanctuary (4900 km²), located in the Little and Great Rann of Kutch Gujarat, is currently the last refugia of
the Asiatic wild ass population in southern Asia. Although construction of the Indian wild ass sanctuary in this salt-impregnated desert has provided shelter to these endangered Asiatic wild asses, but the constant anthropogenic pressure such as salt production, grazing, habitat destruction, and dam construction in and around the sanctuary has threatened their existence (Goyal et al. 1999; Srivastav & Nigam 2010). Recent Gujarat forest reports have confirmed an increase in both population (over 4000) and distribution range of E. h. khur in western India; however, in 1967, a dreaded protozoic disease called Surra caused by Trypanosoma evansi reduced the population of the wild equid to mere 362 individuals (Gujarat Forest Department 1967; Singh 2000; Srivastav & Nigam 2010). Most previous studies on this free-ranging Indian wild ass were confined to demographic or ecological measurements and behaviours without assessing their genetic diversity (Gee 1963; Shah 1999; Shah & Qureshi 2007). The only study that evaluated the genetic diversity of E. h. khur was conducted by Srivastav and Nigam (2010) with few captive individuals from a zoo. Recent improvements in the non-invasive conservation genetics methods have enabled researchers to study the diversity of the elusive and protected wild species worldwide (Beja-Pereira et al. 2009; Allendorf et al. 2010). We conducted the first pilot study for (1) measuring the level of genetic diversity underlying the natural Indian wild ass population in the Little Rann of Kutch, (2) confirming the position of E. h. khur in the ass-like branch of equid phylogeny, and (3) estimating the divergence and demography of these endangered wild asses by using a non-invasive sampling approach.

Materials and methods

Samples

Over 12 months, 52 faecal samples of the free-ranging Indian wild ass E. h. khur were collected from three localities in the sanctuary (Figure 1). The samples were placed in individual zippered bags, dried naturally, and stored at room temperature until further processing. Permission for collecting faecal samples in the wild ass sanctuary was obtained from the state of Gujarat through a letter (No. WLP/28/C/574-76/2013-14; Date: 18/12/2013) issued by the Principal Chief Conservator of Forest (Wildlife), Gujarat.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted using the modified protocol of a spin column tissue DNA extraction kit (GENETIX Miniprep Kit, GENETIX, Rome, GA). Modification to the standard protocol included long-term incubation of the fecal samples in suitable concentrations of lysis buffer, along with the addition of Proteinase K and use of the inhibitEX® tablet (QIAGEN, Valencia, CA) for preventing PCR inhibition. Khurcytb FOR (5'-GAC ACA ACA ACC GCC TTC TC-3') and Khurcytb REV (5'-GAC TGT TGC TCC TCA GAA GGA T-3') were used to amplify approximately 230 bp of the cytochrome b (Cyt b) fragment in 33
samples, whereas KhurDL FOR (5′-TCC CCA TGT GCT ATG TCA GT-3′) and KhurDL REV (5′-GAT ARG CGT GTT GAC TGG AAA-3′) were used to amplify approximately 230 bp of displacement loop (D-loop) in 52 samples. PCR reactions were performed using 20 µL of total reaction mixture composed of 3 µL of deionized water, 8 µL of Dream Taq Mastermix (Thermo Scientific, Waltham, MA), 1.5 µL of 2.5 mM MgCl₂, 1 µL of 0.3% BSA, 0.5 µL (5 U/µL) Taq polymerase (Thermo Scientific, Waltham, MA), 0.8 µM of each primer, and variable amounts of genomic DNA. PCR cycling for both loci was performed in a PCR Verity 96-well thermal cycler (Applied Biosystems, Waltham, MA). For the Cyt b gene, the PCR mixture was subjected to initial denaturation at 94 °C for 15 min, followed by 45 cycles at 94 °C for 50 s, 60 °C for 50 s (annealing), and 72 °C for 45 s (extension), and final extension at 72 °C for 20 min. For D-loop amplification, identical cycling conditions were used, except for the annealing temperature, which was 61.7 °C. After purification with ExoSAP-IT® (Affymetrix), amplicons were sequenced for both strands on an automated DNA sequencer ABI PRISM® 377 (Applied Biosystems, Waltham, MA).

**Molecular phylogeny, dating, and demography**

Chromatogram files were edited and aligned using Geneious 8.0.5 (Applied Biosystems, Waltham, MA). All 33 Cyt b and 52 D-loop partial sequences generated were deposited in Genbank with accession nos. KJ490647–KJ490651 and KT221803–KT221829, KU342004–KU342014, respectively. To clarify the relative positions of E. h. khur D-loop and Cyt b sequences generated using the available wild ass species/subspecies sequences in the database (Supplementary Data), we constructed phylogenetic trees. The best-fit model of evolution was selected using jModelTest (version 2.1.3) (Guindon & Gascuel 2003; Darriba et al. 2012) according to the Akaike information criterion for both D-loop and Cyt b datasets. The phylogenetic trees were inferred using the Bayesian approach as implemented in MrBAYES (version 3.2.1) (Ronquist et al. 2012). To obtain the Bayesian-inferred trees, two independent analyses were performed, and four MCMC chains were run for 20 million generations with sampling at every 1000 generations. After checking convergence, 25% trees were discarded as burn-in. The molecular divergence of E. h. khur was measured using BEAST (version 2.3.1) by estimating time to the most common ancestor among equid species for both datasets (Drummond et al. 2012). A relaxed uncorrelated lognormal clock model and a Yule process of speciation were used. The Asiatic wild ass branch was calibrated using several internal calibration points drawn from the study of Vilstrup et al. (2013) and Rosenbom et al. (2015) for extant equid species. Apart from E. hemionus and E. kiang, monophyly was constrained for species represented in the analyses by more than one individual. While constraining monophyly, normal prior with HKY + G + I and GTR + G + I models were used for Cyt b and D-loop datasets, respectively. Parameters were sampled at every 1000 generations, convergence was viewed using TRACER (version 1.5) (Rambaut & Drummond 2007), and four MCMC chains over a total of 20 million generations were run with 25% generations discarded as burn-in. The program BEAST (version 2.3.1) was used to measure the present and past demography of E. h. khur by using the Bayesian skyline model that estimates the posterior distribution of population sizes (Drummond et al. 2005). A strict molecular clock with normal distribution prior with a clock rate of 3. 6 × 10⁻⁸ per generation, based on the studies of the mitochondrial DNA control region of domestic donkeys and African wild asses (Kimura et al. 2011; Rosenbom et al. 2015) was applied.

**Results**

**Diversity and phylogeny**

The alignment of Cyt b and D-loop regions revealed only one haplotype, indicating low genetic diversity in the extant Indian wild ass population. Homology searching using BLAST 2.2.32 (Zhang et al. 2000; Aleksandr et al. 2008) showed that the E. h. khur haplotype shares 98% identity with the two E. h. kulan sequences (JX312728.1 and JF718886.1). The Bayesian-inferred tree based on Cyt b and D-loop haplotypes demonstrated two well-supported clades within the Asiatic wild ass E. hemionus. In Cyt b haplotype-based BI tree, E. h. khur cluster with E. h. kulan with high posterior support while in another clade which include, Equus kiang, E. h. hemionus and E. h. onager, we failed to recover the relatedness among these species/subspecies owing to resulted polytomy (Figure 2A). In addition, similar to the Cyt b-inferred tree, the tree constructed on the basis of the D-loop sequences also show two well-supported clades, where Indian wild ass, E. h. khur cluster with clade formed by E. h. kulan and E. h. onager while in another well-supported clade, the monophyly of Equus kiang with E. h. hemionus was illustrated (Figure 2B). The measurement of time to the most common ancestor in the presence of the Indian wild ass haplotype demonstrated that the Asiatic wild ass branch diverged approximately 0.65 million years ago (Figure 2B). The estimation of demographic dynamics by using the Bayesian skyline model revealed that the Indian wild ass maintained a steady maternal effective population size (N*e) until 10 kya, after which a population decline was detected (Figure 3).

**Discussion**

The occurrence of a single haplotype in the sampled population strengthens the 1967 Gujarat forest reports, revealing that severe decline in the E. h. khur population in 1962 caused by the protozoic disease Surra in the state. Phylogeny reconstruction of the Asiatic wild ass in the presence of the first-reported Indian wild haplotype is in agreement with the phylogeny inferred by Rosenbom et al. (2015), where two major clades of Asiatic wild asses were identified without any further distinction of subspecies within these clades. These findings were not consistent with the results of other previous studies, where a single monophyletic clade was reported for the entire E. hemionus species/subspecies in which E. kiang haplotypes formed the monophyletic clade within a larger variation of E. hemionus (Steiner et al. 2012; Vilstrup et al. 2013). Therefore, our phylogenetic analyses confirmed that the Indian wild ass, E. h. khur is genetically similar to the endangered E. h. kulan and E. h. onager, commonly called as Turkmenian kulan and Persian onager, respectively. However, the surviving population of approximately 4000 individuals of the Indian wild ass E. h. khur in the Indian wild ass sanctuary is the relic of once
widespread but currently critically endangered population of *E. hemionus* or Persian onager in Asia (http://www.IUCN red-list.org). The successful reintroduction of *E. h. kulan* to Kazakhstan and Uzbekistan during the Soviet Union times, followed by its reintroduction in Israel, where it is forming hybrids with Persian onager in wild, strengthens this finding (Renan et al. 2015). The time depth of 0.65 million years estimated for the Asian wild ass branch is in agreement with the recent studies on wild asses conducted by Rosenbom et al. (2015) and Vilstrup et al. (2013). These results corroborate that climatic events during the Pleistocene were the major driving force in the differentiation processes in wild asses. A decrease in the maternal effective population size of the Indian wild ass approximately after the 10 kya coincides with

**Figure 2.** Molecular divergence estimation using D-loop dataset. Numbers above line are the node ages in Million years, while those below indicate 95% HPD values. Values on nodes indicate posterior probabilities.
the rise of 8000-year-old Neolithic human settlement in Mehrgarh in the northwest of the subcontinent eventually culminating into the 5000-year-old Indus civilization (Possehl Gregory 1996; Kenoyer 1998). However, the expansion of the human population along with hunting and habitat loss must have continued to impose a decreasing trend until recently. A similar decline in effective population sizes has been reported for *E. hemionus* and wild horses that showed a decline in their genetic diversity after the Last Glacial Maximum (LGM) (Der Sarkissian et al. 2015; Rosenbom et al. 2015).

**Conclusion**

By using a non-invasive methodology, we demonstrated that the Indian wild ass *E. h. khur* is genetically similar to *E. h. kulun* and *E. h. onager*, two subspecies of the Asiatic wild ass. Despite the current population of over 4000 individuals, the maternal effective population size of the Indian wild ass has declined, resulting in low genetic diversity. In addition to invoking the revision of systematic of Asiatic wild ass species/subspecies, this pilot study can be a stepping stone for the genetic conservation of the Indian wild ass.

**Acknowledgements**

We thank Albano Beja-Pereira and Sonia Rosenbom, CIBIO, University of Porto, Vairao, Portugal for extending their help in DNA extraction protocol. MB was supported by the Department of Biotechnology, Govt. of India DBT-CREST award 2013-14.

**Disclosure statement**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

**References**

Aleksandr M, George C, Yan R, Thomas LM, Richa A, Alejandro AS. 2008. Database indexing for production MegaBLAST search. Bioinformatics. 24:1757–1764.

Allendorf FW, Hohenlohe PA, Luikart G. 2010. Genomics and the future of conservation genetics. Nat Rev Genet. 11:697–709.

Beja-Pereira A, Oliveira R, Alves PC, Schwartz MK, Luikart G. 2009. Advancing ecological understandings through technological transformations in noninvasive genetics. Mol Ecol Resour. 9:1279–1301.

Darriba D, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 9:772. doi:10.1038/nmeth.2109.

Der Sarkissian C, Ermini LC, Schubert M, Yang MA, Librado P, Fumagalli M, Jónsson H, Bar-Gal GK, Albrechtsen A, Vieira FG, et al. 2015. Evolutionary genomics and conservation of the endangered Przewalski’s horse. Curr Biol. 25:2577–2583.

Drummond AJ, Rabaut B, Shapiro B, Pybus OG. 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. Mol Biol Evol. 22:1185–1192.

Drummond AJ, Suchard MA, Xie D, Rabaut A. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol. 29:1969–1973.

Gee EP. 1963. The Indian wild ass: a survey. J Bomb Nat Hist Soc. 60:517–529.

Goyal SP, Sinha B, Shah N, Panwar HS. 1999. Sardar Sarovar Project – a conservation threat to the Indian wild ass (Equus hemionus khur). Biol Cons. 88:277–284.

Groves CP. 1974. Horses, asses and Zebras in the wild. Hollywood, Florida, USA: Ralph Curtis Books. p. 192.

Groves CP, Mazák V. 1967. On some taxonomic problems of Asiatic wild asses; with the description of a new subspecies (*Perissodactyla; Equidae*). Z Saugetierkunde. 32:321–355.

Grubb P, editor. 2005. Order Perissodactyla. Baltimore (MD): The Johns Hopkins University Press.

Guindon S, Gascuel O. 2003. A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. Syst Biol. 52:696–704.

Kenoyer JM. 1998. Ancient cities of the Indus Valley Civilization. Oxford: Oxford University Press. p. 38.

Kimura B, Marshall FB, Chen S, Rosenbom S, Moehlman PD, Tuross N, Sabin RC, Peters J, Barich B, Yohannes H, et al. 2011. Ancient DNA from Nubian and Somali wild ass provides insights into donkey ancestry and domestication. Proc Biol Sci. 278:50–57.

MacFadden BJ. 2005. Evolution: fossil horses-evidence for evolution. Science (New York, NY). 307:1728–1730.

McCue ME, Bannasch DL, Petersen JL, Gurr J, Bailey E, Binns MM, Distl O, Guerin G, Hasegawa T, Hill EW, et al. 2008. A high density SNP array for the domestic horse and extant Perissodactyla: utility for association mapping, genetic diversity, and phylogey studies. PLoS Genet. 8:e1002451.
Moehlman PD, editor. 2002. Equids: zebras, asses and horses. Status survey and conservation. Gland, Switzerland: IUCN.

Oakenfull EA, Lim H, Ryder O. 2000. A survey of equid mitochondrial DNA: implications for the evolution, genetic diversity and conservation of Equus. Conserv Genet. 1:341–355.

Possehl Gregory L. 1996. Mehrgarh. In: Fagan B, editor. Oxford companion to archaeology. Oxford: Oxford University Press.

Rambaut A, Drummond AJ. 2007. Tracer Version 1.4. Available from: http://tree.bio.ed.ac.uk/software/tracer/.

Ronquist F, Teslenko M, vander Mark P, Ayres DL, Darling A, Höhna S, Lartet B, Liu L, Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol. 61:539–542.

Renan S, Greenbaum G, Shahar N, Templeton A, Bouskila A, Bar-David S. 2015. Stochastic modeling of shifts in allele frequencies reveals a strongly polygynous mating system in the re-introduced Asiatic wild ass. Mol Ecol. 24:1433–1446.

Rosenborn S, Costa V, Chen S, Khalatbari L, Yusefi G, Abdakadir A, Yangzom C, Kebede F, Teclai R, Yohannes H, et al. 2015. Reassessing the evolutionary history of ass-like equids: insight from pattern of genetic variation in contemporary extant populations. Mol Phylogenet Evol. 85:88–96.

Ryder OA, Chemnick LG. 1990. Chromosomal and molecular evolution in Asiatic wild asses. Genetica. 83:67–72.

Schaller GB. 1998. Wild life of the Tibetan Steppe. Chicago (IL): University of Chicago Press.

Shah N. 2002. Status and action plan for the Kiang (Equus kiang). In: Moehlman PD, editor. Status survey and conservation action plan – equids: zebras, asses and horses, gland. Switzerland: IUCN.

Shah NV. 1999. Mammals. In: Singh HS, Patel BH, Parvez R, Soni VC, Shah N, Tatu K, Patel D, editors. Ecological study of wild ass sanctuary little Rann of Kutch. Gandhinagar, Gujarat, India: GEER Foundation.

Shah NV, Qureshi Q. 2007. Social organization and determinants of spatial distribution of khur (Equus hemionus khur). Explor Biol Resourc Mongolia. 10:189–2000.

Singh HB. 2000. Status of Indian wild ass (Equus hemionus khur) in the little Rann of Kutch. Zoos Print. 15:253–256.

Srivastav A, Nigam P. 2010. National studbook of Indian Wild Ass (Equus hemionus khur). New Delhi: Wild life Institute of India, Dehradun and Central Zoo Authority.

Steiner CC, Mitelberg A, Tursi R, Ryder OA. 2012. Molecular phylogeny of extant equids and effects of ancestral polymorphism in resolving species-level phylogenies. Mol Phylogenet Evol. 65:573–581.

Steiner CC, Ryder OA. 2011. Molecular phylogeny and evolution of the Perissodactyla. Zool J Linn Soc. 163:1289–1303.

Vilstrup JT, Seguin-Orlando A, Stiller M, Ginolhac A, Raghavan M, Nielsen SC, Weinstock J, Froese D, Vasiliev SK, Ovodov ND, et al. 2013. Mitochondrial phylogenomics of modern and ancient equids. PLoS One. 8:e55950.

Zhang Z, Schwartz S, Wagner L, Miller W. 2000. A greedy algorithm for aligning DNA sequences. J Comput Biol. 7:203–214.