Genetic characterization of free-ranging Asiatic wild ass in Central Asia as a basis for future conservation strategies

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Abstract

Loss of genetic diversity due to drift and inbreeding reduces a population's ability to respond to environmental change and may result in inbreeding depression. The Asiatic wild ass (*Equus hemionus*), regionally also known as Gobi khulan, Turkmen kulan, or Persian onager, has become confined to less than 3% of its historic distribution range. Remaining populations in Central Asia outside of the Mongolian Gobi are small and fragmented. Questions concerning subpopulation status remain disputed and concerns over the viability of these populations have been raised because of small size, past bottlenecks, or recent founder events. We used non-invasive faecal samples to assess the genetic diversity and divergence among Turkmen kulan and Persian onager from five free-ranging and one captive population from Turkmenistan, Kazakhstan and Iran and compared their genetic constitution to the large autochthonous population in the Mongolian Gobi. We observed loss of genetic diversity (drift and inbreeding) in the captive and reintroduced populations as well as in one rapidly declining autochthonous population. Population differentiation and structure using microsatellites and mtDNA based phylogenetic analysis do not support the current separation of the autochthonous populations of Turkmen kulan and Persian onager into different subspecies, but rather suggest a cline with the Iranian population in Bahram-e-Goor at the southern end and the Turkmen population in Badhyz at the northern end falling into two distinct clusters, and the northern Iranian population in Touran being intermediate. We compare our findings to other population genetics studies of equids and discuss the implications of our findings for the future conservation of the Asiatic wild ass in the region.

Key words: Asiatic wild ass; *Equus hemionus*; microsatellites; mtDNA; inbreeding; reintroduction

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Introduction

Small populations face a higher extinction risk due to the combined effect of demographic, environmental, and genetic stochasticity (Brook et al. 2002). With shrinking population size mate choice becomes restricted, eventually leading to mating between close kin, resulting in inbred offspring. Inbreeding has been documented to reduce survival and reproduction, making inbreeding depression a major concern in small populations (Armbruster and Reed 2005; Armstrong and Cassey 2007). The loss of genetic diversity due to drift and inbreeding also reduces a population's ability to respond to environmental change or to cope with new parasites or pathogens (Frankham 2003). Large natural populations may have a low genetic variability because of a past population bottleneck (Hoelzel et al. 2002; Kuehn et al. 2003, 2004) or a small number of founders, the latter often being the case in reintroduced populations (Jamieson 2011; Tracy et al. 2011).

The Asiatic wild ass (*Equus hemionus*) has become confined to less than 3% of its historic distribution range. By the mid-20th century autochthonous populations only survived in Mongolia, northern China, India, Turkmenistan, and Iran. Except for the large and continuous population in the Mongolian Gobi, all other wild ass populations are small, fragmented and/or have undergone severe population bottlenecks (Kaczensky et al. 2015). The taxonomic status of the Asiatic wild asses has not been fully resolved and recent phylogenetic analysis is increasingly challenging the current systematic subdivision of the Asiatic wild asses into two species (*E. hemionus* and *E. kiang*) and four subspecies (Rosenbom et al. 2015; Bennett et al. 2017; Khaire et al. 2017). However, even if eventually considered one species, their occurrence in such diverse ecological zones as the Himalayan plateau, the deserts of central Asia and the coastal grasslands of India, has very likely resulted in important local adaptations (e.g. see Librado et al. 2015) that justifies assignment to Evolutionarily Significant Units (ESUs) or at least Management Units (MUs) (Funk et al. 2012). Following the precautionary principle, the maintenance of regional genetic variability and differentiation should be considered for captive breeding or reintroduction programs, particularly in the light of anthropogenic impact (Kuehn et al. 2007), host-

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99 parasite interactions (Geist and Kuehn 2008), newly emerging diseases (Pearman and Garner 2005)
100 and global climate change (Luo et al. 2015; Rubidge et al. 2012).
101 Two of the four presently recognized subspecies are of particular conservation concern and are listed
102 as "Endangered" by the IUCN Red List of Threatened Species: the Turkmen kulan (*Equus hemionus*
103 *kulan*; Kaczensky et al. 2016a) and the Persian onager (*Equus hemionus onager*; Hemami et al. 2015).
104 The Turkmen kulan became restricted to the southern parts of Turkmenistan by the 1930s. In 1941,
105 the Badhyz State Nature Reserve was established to protect this remaining population, then estimated
106 at around 150-200 animals (Bannikov 1981). With numbers recovering, the Badhyz population became
107 a source for reintroductions to other parts of Turkmenistan, several locations in Kazakhstan, and
108 international captive breeding facilities (Kaczensky et al. 2016b; Lukarevski and Gorelov 2007; Volf
109 2010). Although reintroductions were initially successful in several locations and increased the
110 Turkmen kulan's range (Kaczensky et al. 2016b), the most recent population estimates for
111 Turkmenistan suggest that the autochthonous and almost all reintroduced populations have
112 dramatically declined to the brink of extinction, most likely due to illegal hunting (Kaczensky and Linnell
113 2015).
114 The reintroduced population in Altyn Emel National Park in Kazakhstan grew rapidly following
115 reintroduction and despite having been subject to a series of bottlenecks. The population was
116 established with 32 founders from another reintroduced population established 20 years earlier on
117 Barsa Kelmes Island in the Kazak part of the Aral Sea (Pavlov 1996). The Altyn Emel reintroduction was
118 so successful that it now constitutes the single largest population of Turkmen kulan, estimated to
119 number >3000 individuals (Fig S1a). The population in Altyn Emel is believed to be close to its habitat's
120 carrying capacity and interest in providing kulan for further reintroductions elsewhere in Kazakhstan
121 is high (Kaczensky et al. 2017; Levanov et al. 2013).
122 The Persian onager disappeared from western Iran in the 1930s but was still widespread in the central
123 and eastern arid and semiarid plains until the 1950s (Denzau and Denzau 1999). By the 1980s, the

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onager had become confined to only four populations, and by the 2000s to only two populations: one in the Touran protected area complex in northeastern Iran and one in Qatrouiyeh National Park and the adjacent Bahram-e-Goor protected area in south-western Iran (Kaczensky et al. 2015, Fig S1b). Both populations are small, with the highly threatened Touran population estimated at 150 individuals and the Bahram-e-Goor population estimated at 685 individuals in 2015 (Hamidi et al. 2012; Hemami et al. 2012; Hemami and Momeni 2013; Iranian Department of Environment (DoE) unpublished data 2015). Because of the precarious status of the wild populations, the Gourab breeding center near Yazd was established in 1997 (Hamadanian 2005). Unfortunately, the entire captive breeding stock descends from four wild-caught founders from Touran and poor reproductive success after initial population growth suggested inbreeding depression.

Concerns over the genetic viability of the remaining free-ranging Turkmen kulan and Persian onager populations have been raised because of their small size, past bottlenecks, or founder events. So far, population-level genetic analysis of free-ranging Asiatic wild ass populations has been restricted to Mongolia and Israel. The large autochthonous population in the Mongolian Gobi shows high levels of genetic variability and low levels of differentiation (Kaczensky et al. 2011). Similar low values of structuring for a large herbivore have only ever been described for Plains zebra (*Equus quagga*; Lorenzen et al. 2008, also see Table S1). The small but expanding reintroduced wild ass population in Israel, on the other hand, has a much lower genetic diversity and shows a very distinct population structure believed to be the result of successive colonization and founder effects (Gueta et al. 2014) and the polygynous mating system (Renan et al. 2015). Evaluating the long term genetic effects of reintroduction and captive breeding is often hampered by the fact that most of these interventions are relatively recent. The Asiatic wild ass example allows us to study the effects of conservation interventions, some of which were taken more than 60 years ago, providing unique insights into the genetic consequences of these actions.

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To assess the genetic diversity of, and divergence among, Turkmen kulan and Persian onager we genotyped non-invasive samples from five free-ranging populations from Turkmenistan, Kazakhstan and Iran and compared their genetic constitution with that of the large autochthonous population in the Mongolian Gobi (Kaczensky et al. 2011) and the highly inbred captive population of Persian onager from the Gourab breeding center in Iran. We also sequenced a segment of the hypervariable region 1 (HVRI) mtDNA for six individuals in each of the remaining autochthonous Turkmen kulan and Persian onager populations and inferred phylogeny including previously published Asiatic wild ass sequences (Bennett et al. 2017; Oakenfull et al. 2000; Rosenbom et al. 2015). Given the recent history of these Central Asian kulan populations, we expected to see a relatively high variability in the autochthonous populations, but loss of genetic variability in the two reintroduced populations (Gury Howdan in Turkmenistan, Altyn Emel in Kazakhstan), and the inbred captive population (Gourab in Iran). Based on the historic distribution of wild asses in the region, we additionally expected the Touran population to be more similar to the Badhyz than to the Bahram-e-Goor population, and all three populations to differ from the Gobi population. Based on previously published results we expected haplotypes to fall into two main clades (Persian onager and Turkmen kulan versus Gobi khulan and Kiang *E. kiang*) but with an additionally subdivision into a Persian onager and Turkmen kulan sub-clade in line with our microsatellite allele frequencies. We compare our findings to other population genetics studies of equids and discuss the implications for the future conservation of the Asiatic wild ass in the region.

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Methods

Study areas and sampling

We collected non-invasive genetic samples in the form of fresh dung in two areas in Turkmenistan, one area in Kazakhstan, and three areas in Iran (Fig. 1). All areas are located in semi-desert and desert-steppe habitats of the Irano-Touranian biogeographic province (Udvardy 1975). For climatic site characterization, we used the WorldClim data from Hijmans et al. (2005).

Badhyz State Nature Reserve (Turkmenistan; TB) was established in 1941 and covers 1,400 km²; though including the buffer zone, adjacent wildlife sanctuaries, and two wildlife corridors the total protected area network amounts to 2,893 km². Elevations range from 300 to 1,200 m, average annual temperature is 15°C and average annual precipitation 250mm. Kulan numbers reached a low in the 1950s but eventually recovered, reaching peak numbers in the 1990s. After the breakdown of the Soviet Union, numbers dramatically declined due to high levels of illegal killings. This trend was reversed again in the early 2000s, when improved protection lead to a renewed increase. Most recent estimates suggest that the population has dramatically decreased and is likely at the brink of extinction (Kaczensky and Linnell 2015, Fig. S1a). We collected 33 dung samples from 10 different locations, mainly around water points, in October 2014.

The 150 km² Gury Howdan Wildlife Sanctuary (Turkmenistan; TG) is located in the northern foothills of the Kopet Dag Mountains, some 20 km southeast of the capital Ashgabat. Elevations range from 350 to 1,800 m, average annual temperature is 14°C and average annual precipitation 260mm. In 1981, a total of 11 kulan were reintroduced originating from Badhyz; an additional seven, also originating from Badhyz, were released in nearby Kalinin (Fig. 1). The population grew during the first 15 years but has decreased thereafter and is currently estimated at 13 animals in two groups. Kulan primarily use the sanctuary's buffer zone and their habitat is increasingly encroached by tree-plantations, agriculture, and livestock grazing. We collected 10 dung samples from one group of animals seen on a large stubble field in September 2015.

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193 Altyn Emel National Park (Kazakhstan; AE) was designated in 1996 and covers 4,600 km² plus an
194 additional 600 km² buffer zone. Elevations range from 500 to 2,900 m, average annual temperature is
195 9°C and average annual precipitation is 630mm. From 1982-1984 a total of 32 kulan were reintroduced
196 originating from the reintroduced population on Barsa Kelmes (BKI), an island in the former Aral Sea
197 (Fig. 1). The population in Altyn Emel grew rapidly and is currently estimated at >3,000 animals (K.
198 Bayadilov unpubl. data 2015). Kulan from Altyn Emely have been used as a source for re-introductions
199 to the Andassay Sanctuary (Levanov et al. 2013). We collected 81 dung samples from seven different
200 locations in October 2014.
201 Bahram-e-Goor (Iran; IB) protected area covers 4,080 km², which includes the 318 km² Qatrouiyeh
202 National Park. The protected area was established in 1972 and is located in the southern part of the
203 Iranian plateau. Elevations range from 1600 to 2500 m, the average annual temperature is 15°C and
204 average annual precipitation 150mm. The onager population was at its lowest point in 2000 at an
205 estimated 85-90 animals (Hamadanian 2005) but has shown a positive trend in recent years and was
206 estimated at 685 in 2015 (L. Joulaie (DoE) unpubl. data). We collected 18 dung samples from three
207 different locations in July 2011.
208 Touran protected area network (Iran; IT) covers some 14,649 km² in northeastern Iran and was
209 established in 1975. Elevations range from 700 to 2200 m, average annual temperature is 17°C and
210 average annual precipitation 160mm. The population was estimated at 600-700 in the early 1970s but
211 declined sharply thereafter (Hamadanian 2005; Tatin et al., 2009) and was estimated at around 150 in
212 2015 (M. Adibi (DoE) unpubl. data). We collected six dung samples from four regions throughout the
213 protected area in July and August 2012.
214 The Gourab breeding center (Iran; IG) near Yazd was established with four wild caught animals (two
215 males and two females) from Touran in 1997 (Hamadanian 2005). Until 2007, the captive population
216 in the 133 ha enclosure reached 32 individuals, and subsequently animals from Gourab were used to
217 establish another seven breeding centers. In 2014, the Gourab breeding center was supplemented

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with four animals captured in Touran. As of 2015, there were 54 wild asses in various captive facilities of which 20 were in Gourab (B. Shahriari (DoE) unpubl. data). We collected eight dung samples from the Gourab breeding center in July 2011 when the captive population numbered 10 animals. For a more detailed description of kulan numbers in the six Central Asian populations see Fig S1a,b.

To evaluate the genetic constitution of the Central Asian wild ass samples we compared them to the large population in the Mongolian Gobi based on 80 samples from 3 regions previously published in Kaczensky et al. (2011; 19 samples from the Dzungarian Gobi (GGB), 18 samples from the Transaltai Gobi (GGA), and 43 samples from the Southern Gobi (GGS)). In the three regions of the Mongolian Gobi elevations range from 550 to 3800 m, average annual temperature from 1-7°C and average annual precipitation from 50-200mm. The Mongolian population is currently estimated at around 42,000 individuals (Kaczensky et al. 2015). For more details on sampling and a detailed site characterization see Kaczensky et al. (2011).

DNA extraction, microsatellite genotyping, and mtDNA analysis

To avoid DNA cross-contamination between samples, we only collected one pellet per dung pile and only from dung piles which had no physical contact with other dung piles. We extracted fecal DNA with a slightly modified protocol of the QIAampH DNA Stool MiniKit (QIAGEN, Hilden, Germany) and eluted fecal DNA samples in 80 µL as described in Kaczensky et al. (2011, sup. material) and Hausknecht et al. (2010).

We quantified DNA concentration of extracts by qPCR using a SYBR Green–based assay in a LightCycler 1.0 Instrument (Roche Applied Science, Mannheim, Germany). For species verification, we applied restriction fragment length polymorphism analysis of the mitochondrial cytochrome b gene fragment (Kuehn et al. 2006). We used nine microsatellite loci (COR70, SGCV28, ASB23, COR58, LEX68, COR18, UM11, COR007, and COR71) established for wild asses by Kaczensky et al. (2011) and performed multiplex PCRs in a total volume of 15.0 µl containing 3.5 µl template DNA using the QIAGEN Multiplex PCR Plus Kit (QIAGEN, Hilden, Germany). Multiplex PCR reactions were performed on an Mastercycler

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Gradient Thermal cycler (Eppendorf) with the primer combination COR70, SGCV28, ASB23, COR58 and COR007 for the multiplex system I and LEX68, COR18, UM11, and COR71 for the multiplex system II using the annealing temperature of 60°C and 58°C, respectively. The forward primers were fluorescently end-labeled with Tamra, Hex, and 6-Fam for genotyping on 6% polyacrylamide gels on an ABI Prism 377 automated sequencer (Applied Biosystems).

We scored the alleles in reference to a ROX standard (GS 79–362 bp; DeWoody et al. 2004) by GENESCAN® 3.1.2 and GENOTYPER® 2.5 software (Applied Biosystems, Foster City, CA, USA). To ensure genotyping reliability, and misinterpretations of microsatellite data due to allelic drop-out and false alleles (Taberlet and Luikart 1999), we included an analysis quality management system as described in Kaczensky et al. (2011): negative controls without sample material in every DNA isolation and amplification experiment, excluding of samples with less than 100 pg/µl, genotyping analyses in triplicate, and rejection of samples with the same multi locus genotype. Of our 156 dung samples from Iran, Turkmenistan, and Kazakhstan, 138 (88.5%) passed the quality criteria and showed a unique genotype; and together with the 80 samples from the Mongolian Gobi make for an overall sample size of 218.

In addition to nuclear markers, we used the hypervariable region 1 (HVRI) of the mtDNA control region (440bp) to investigate the phylogeny of the Persian onager, Turkmen kulan and Gobi khulan / Kiang (*Equus kiang*). PCR was performed in a total volume of 25 µl composed of 0.2 µM of each primer, (forward primer: CCCAAGGACTATCAAGGA reverse primer: GGAATGGCCCTGAAGAAA G (Rosenbom et al. 2015)), 0.2 mM of each dNTP (Solis BioDyne, Tartu, Estonia), 3 mM MgCl2 (Solis BioDyne,), 1× PCR buffer (Solis BioDyne), and 0.5 U Taq DNA Polymerase (FIREPol®, Solis BioDyne), and variable amount of genomic DNA. PCR was carried out on a Mastercycler Gradient Thermal cycler (Eppendorf) with cycling conditions as follows: initial denaturation at 94 °C for 3 min; 35 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, before final extension at 72 °C for 3 min. We purified PCR products using a NucleoSpin Extract Kit (Macherey and Nagel, Düren, Germany) and sequenced in both directions at

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Sequiserve GmbH (Vaterstetten, Germany). We submitted our sequences to GenBank (preliminarily accession numbers MH102360 – MH102377).

Data Analysis

We used FSTAT v. 2.9.3 (Goudet 2001) for calculating allele frequency, average number of alleles per locus (*A*), expected heterozygosity (H_E), observed heterozygosity (H_0), inbreeding coefficient (F_{IS}) and allelic richness (A_R) as a standardized measure of the number of alleles (corrected for sample size) and to calculate pairwise F_{ST}. In some scenarios (e.g. very high genetic diversity) assumptions of the population genetic structure based on F_{ST} may be misleading. Hence, we also calculated Jost's measure of estimated differentiation (D_{EST}) (Jost 2008) using GenAlEx v. 6.5 (Peakall and Smouse 2012). The latter was also used to identify private alleles (>5% occurrence) (A_{priv}). The analysis of molecular variance (AMOVA; Excoffier et al. 1992) was carried out with ARLEQUIN v. 3.1 (Excoffier et al. 2005). We calculated the mean individual inbreeding coefficient (F_{ind}) per population by computing the likelihood of the homozygote state p(homozygote) = F + (1− F)Σpi2 , were i represents the alleles and pi the frequency of allele i in a given genotype. We summed up the log-likelihood across loci for multilocus genotypes, using the adegenet R package (Jombart 2008). We calculated linkage disequilibrium and conformance with Hardy–Weinberg expectations (P_{HW}) in Genepop on the Web v 4.2 (Raymond and Rousset 1995) using the probability test with Markov chain parameters, 1000 dememorizations, 100 batches and 1000 iterations per batch. Since all populations deviated from Hardy–Weinberg proportions after Bonferroni correction, we did not check for the presence of null alleles due to the difficulty of assessing heterozygote deficit versus null alleles (David et al. 2007). We used the program MICRO-CHECKER v.2.2.3 (van Oosterhout et al. 2004) to identify potential genotyping errors. Following recommendations of Peery et al. (2012) we used the M-ratio test (Garza and Williamson 2001) to assess whether populations underwent a recent reduction in effective population size, using the critical value of 0.68 as recommended by Garza and Williamson (2001). We additionally tested for evidence of a recent reduction in effective population size using Wilcoxon sign-

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293 rank test as implemented in the BOTTLENECK computer programme (Cornuet and Luikart 1996),
294 assuming the stepwise model (SMM), the infinite allele model (IAM) and the two-phase model of
295 mutation (TPM). We used STRUCTURE 2.3.4 software (Pritchard et al. 2000) to reveal and visualize the
296 genetic structure of the Irano-Touranian and Mongolian wild ass populations, determine the number
297 of genetic clusters (K), and assign the probabilities of an individual belonging to each cluster. We tested
298 the number of clusters from 1 to 10 with 10 iterations for each K (200 000 burn-ins, 1 500 000 Markov
299 chain Monte Carlo replicates in each run) using the admixture model and assuming correlated allele
300 frequencies to assess convergence of the probability ln P(X|K). We determined the final number of
301 clusters from ΔK, the rate of change in the log probability over all 10 iterations (Evanno et al. 2005)
302 using STRUCTURE HARVESTER (Earl and von Holdt 2012). We used CLUMPP software (Jakobsson and
303 Rosenberg 2007) with the 'greedy' option with 10000 random input orders to find the optimal
304 individual alignments of replicated cluster analyses and calculated the average cluster portions within
305 populations.
306 Because Bayesian clustering techniques may produce biased results when faced with unequal sample
307 sizes (Puechmaille 2016), we verified the result of STRUCTURE with the multivariate Discriminant
308 Analysis of Principal Components approach (DAPC; Jombart et al. 2010), which is less sensitive when
309 sampling is uneven (Puechmaille 2016). DAPC was implemented in the ADEGENET package in R Version
310 3.2.3. This method firstly extracts information by applying a principal component analysis (PCA) and
311 secondly maximizes the between-group component of the genetic variation using a discriminant
312 analysis (DA). In the first step of this procedure 40 principal components of PCA were retained which
313 explained approximately 85% of the total variation in the data set. The result of the DAPC were
314 visualized by RGB color coding (color is based on assignment values of each individual to the first three
315 principal components multiplied by 255); the similarity of the dot color represents the genetic
316 similarity of the populations and individuals (Jombart 2008, Jombart et al. 2010). We calculated the
317 heterozygosity contribution (CT) and allelic richness contribution (CTR, data not shown) of each

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318 population to total diversity with the CONTRIB program (Petit et al. 1998) by separately calculating
319 diversity and differentiation indices measured by the expected heterozygosity and allelic richness. This
320 approach allows a simultaneous comparison of a populations' diversity and differentiation
321 contribution and supplements the genetic characterization of populations and the selection of priority
322 populations for conservation.
323 We inferred the phylogenetic relationships among newly obtained HVRI-haplotypes and previously
324 published Asiatic wild ass sequences (AF220934-AF220937 see Oakenfull et al. 2000; KP825311-
325 KP825326 see Rosenbom et al 2015; KY749129-KY749144; KY749164-KY749181 see Bennett et al.
326 2017) using the MEGA7 (Kumar et al. 2016) software. We aligned and trimmed sequences using the
327 DECIPHER R-Package (Wright 2015) and Maximum Likelihood (ML) method to determine the best
328 substitution model and construct the phylogenetic tree using the best-fit model based on the Bayesian
329 Information Criterion approach (T92+G, Tamura 3-parameter, Tamura (1992)) and the Nearest-
330 Neighbor-Interchange (NNI) as the ML heuristic search method. The reliability of the phylogenetic tree
331 was assessed by bootstrap sampling strategy with 1000 replications.
332

333 # Results

334 **Linkage and Hardy–Weinberg equilibrium**

335 The test for genotypic disequilibrium for each pair of the nine microsatellite loci over all populations
336 gave no significant value for 45 comparisons after Bonferroni correction for multiple tests. When each
337 population was tested separately, a linkage equilibrium between all pairs of loci was generally
338 observed, with only a few exceptions: three significant values were found but different loci were
339 involved in these cases. Generally, this test implies that the genotypes of the loci used in this study
340 segregated independently. All populations displayed significant deviations from the expected Hardy–
341 Weinberg equilibrium (HWE) after applying sequential Bonferroni correction (see Table 1). The Altyn
342 Emel population displayed a significant deviation at all loci, Badhyz at seven loci and Gourab at four

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loci; this suggests some form of substructure within these populations which is in concordance with the results of the structure analysis (Fig. 4).

Genetic diversity

The expected heterozygosity (H_E) ranged from 0.598 in Bahram-e-Goor to 0.843 in Great Gobi B and was 8-28% lower in the six populations of Turkmen kulan and Persian onager as compared to the mean of the three subpopulations from the Mongolian Gobi (Table 1). The observed heterozygosity (H_0) - with a range from 0.177 in Gourab to 0.776 in Great Gobi B was dramatically lower than the expected heterozygosity in all three populations of Turkmen kulan (Badhyz, Altyn Emel, Gury Howdan) and somewhat lower in Bahram-e-Goor, one of the three Persian onager populations. The most extreme difference was observed in the captive population of Gourab (H_E = 0. 651, H_0 = 0.177, Table 1, Fig. 2A). Allelic richness ranged from 3.4 to 5.7 and was lowest for Gourab, Bahram-e-Goor, and Gury Howdan, intermediate for Badhyz, Touran, and Altyn Emel, and highest for the Mongolian Gobi populations. The population level inbreeding coefficient F_{IS} (range: 0.081 to 0.745) and the individual inbreeding coefficients F_{ind} (range: 0.197 to 0.597) were extremely high in Gourab, high in Gury Howdan, Badhyz, and Altyn Emel, and low in Bahram-e-Goor, Touran and the Mongolian Gobi (Table 1, Fig. 3).

Of a total of 151 alleles, twelve (8%) only occurred in one of the nine populations/subpopulations (private alleles), varying from none in Altyn Emel to a maximum of three in Great Gobi A (Table 1). The heterozygosity contribution CT of each population to total diversity is visualized in Fig. S2; the highest diversity contributions stem from the population in the Mongolian Gobi, whereas the populations from Badhyz and Touran added little additional diversity. The reintroduced populations in Altyn Emel, and Gury Howdan as well as the captive population in Gourab showed negative values for diversity contribution.

The M-ratio test revealed evidence for recent bottlenecks in the reintroduced Gury Howdan and the captive Gourab population (Table 1). The Wilcoxon sign-rank test ($P < 0.05$) showed substantial recent bottlenecks for all Turkmen kulan and Persian onager populations based on the IAM and SMM model,

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368 whereas the TPM model pointed towards recent bottlenecks in the Gury Howdan, Bahram-e-Goor,
369 Gourab, and Touran, but not in the Altyn Emel and Badkyz populations.

370 **Genetic differentiation between populations and phylogenetic relationship**

371 The degree of genetic differentiation based on F_{ST} values was low between the three Mongolian Gobi
372 populations with an average F_{ST} value of 0.01 (SD = 0.006). Differentiation between the Gobi
373 populations and those in Badhyz and Touran showed moderate differentiation with F_{ST} values of 0.084-
374 0.101 and 0.063-0.077, respectively. As expected, Touran revealed a low differentiation to the captive
375 population of Gourab (F_{ST} = 0.051), and Badhyz a low differentiation to the reintroduced Altyn Emel
376 population (F_{ST} = 0.045). All other populations displayed a substantial degree of genetic differentiation;
377 the second highest value of differentiation (F_{ST} = 0.1248) was found between the two autochthonous
378 Iranian populations in Bahram-e-Goor in the south and Touran in the north (Table 2).
379 D_{EST} values ranged from 0.009 to 0.539 and showed a significant correlation (r^2 = 0.718, P < 0.0001)
380 with the F_{ST} -values. The Mantel test found no significant correlation between geographic distance and
381 genetic differentiation of the populations (F_{ST}, r^2 = 0.001, P = 0.840), though there was a significant
382 correlation between the geographic distance and the Jost value (D_{EST}, r^2 = 0.131, P = 0.029). However,
383 F_{ST} is a more important summary of the effects of population structure than D_{EST} (Whitlock 2011).
384 AMOVA of hierarchical gene diversity revealed that 67% of the genetic variation was accounted for
385 within individuals, 25% among individuals within populations, and 8% between populations. The global
386 fixation indices were 0.271, 0.084, and 0.333 for F_{IS}, F_{ST}, and F_{IT}, respectively.
387 The result of the DAPC analysis is shown in Figure 2. The first three DA eigenvalues showed 42%, 23%,
388 and 13% of the retained variation, respectively. The color of each population and individual,
389 represented by a dot, corresponds to the discriminant components, which are recoded in color
390 channels of the RGB system. The discriminant analysis of the principal components resulted in a close
391 clustering of Turkmen kulan from Badhyz and Altyn Emel, whereas the Gury Howdan population
392 already showed clear signs of drift from its source (Fig. 2A und B). The Persian onagers from Gourab

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still clustered with their source population in Touran, but also revealed signs of drift. The Persian onager from Touran were more similar to the Turkmen kulan and Gobi khulan than to the Persian onager from Bahram-e-Goor. Whereas the clusters of Turkmen kulan, northern Iranian onager (Touran) and Gobi khulan were close and overlapped, there was no overlap with the southern Iranian onager from Bahram-e-Goor (Fig. 2B). Color coded genetic characterization of individuals and populations/subpopulations based on principal component analysis clearly depicts: i) the similarity between the Badhyz-Altyn Emel and Touran-Gourab individuals, ii) the difference of the Bahram-e-Goor individuals, and iii) the high genetic diversity of, and the great similarity among, the Gobi subpopulations. But it also shows that the Gury Howdan population is already rather distinct from its source in Badhyz (Fig. 2C) with signs of loss of genetic variability (Fig. 2, Table 1).

Structure analysis identified the most likely subdivisions to be 2, 3, or 4 genetic clusters K (Fig. 4). The Mongolian populations fall into one (“grey”) cluster and show little internal sub-structuring even when choosing four clusters. The Turkmen kulan population from Badhyz and its derivative populations of Altyn Emel and Gury Howdan fall into the same (“orange”) cluster at K=3 but reveal the effect of small founders and drift at K=4 (“yellow” cluster). For the three Iranian populations, the differentiation between Bahram-e-Goor (“blue cluster”) and Touran (intermediate between “blue” and “orange” cluster) becomes increasingly apparent with increasing K, as does Touran being the source for Gourab (Fig. 4). These patterns are consistent with the results of genetic divergence and distance (Tab. 2) and the results of the DAPC (Fig. 2).

ML analysis of newly sequenced Persian onager and Turkmen kulan HVRI haplotypes from the remaining autochthonous population and reanalyzed with previously published Asiatic wild ass sequences shows two differently supported clades, a well-supported Gobi khulan and kiang group and a less supported Persian onager and Turkmen kulan group which is polyphyletic interspersed by a Gobi khulan and a kiang splinter group (Fig. 5). A clear subdivision based on the origin of animals from the three remaining autochthonous populations in Bahram-e-Goor, Touran, or Badhyz is not supported.

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Discussion

Genetic consequences of recent population interventions

Genetic differentiation among the Turkmen kulan, Persian onager, and Gobi khulan populations/subpopulations reflect both their geographic location and their recent conservation status which has been heavily influenced by human interventions (e.g. the deliberate transfer of animals over long distances of up to 1600 km within the course of reintroductions (Table S3)). Having been separated for 50-60 years (7-8 generations; assuming a generation time of 7.5 years; Ransom et al. 2016) from its source in Badhyz, the large Altyn Emel population - despite being the result of two successive founder events – shows little sign of drift and has retained much of the original diversity, though several individuals have moderate to high inbreeding coefficients. The small Gury Howdan population, having only been separated from its source in Badhyz for 30 years (4 generations), on the other hand, shows clear signs of drift and loss of genetic diversity. The same trend can already be observed after 14 years (2 generations) of captive breeding in Gourab with only four founders from Touran. This illustrates how intense drift acts on small, isolated populations, particular those originating from a small number of founders, and highlights the importance of discussing and evaluating the genetic constitution of populations in the context of their biogeographic and demographic history (Frankham 1995).

Regional differentiation - Turkmen kulan or Persian onager?

The autochthonous population of Persian onager in Bahram-e-Goor, southern Iran, was most distant from all other populations, contributed most to overall genetic differentiation, and at the same time showed little evidence of genetic drift. The geographically closest autochthonous population in Touran, northern Iran, remained genetically diverse despite its small size and showed little evidence of genetic drift; the same is true for the autochthonous population of Turkmen kulan in Badhyz, southern Turkmenistan. The relative high diversity of Bahram-e-Goor, Touran and Badhyz, despite past or

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present bottlenecks, may be the consequence of being close to the evolutionary cradle of the species (Bennett et al. 2017).

As expected from their biogeographic location, our results do not support the current subdivision of the Asiatic wild ass into Turkmen kulan and Persian onager based on national borders, but point towards a cline from a northern Turkmen kulan cluster (Badhyz) to a southern Persian onager cluster (Bahram-e-Goor), with the Touran population in northern Iranian being somewhat intermediate. Natural barriers, like the Zagros and central Iranian mountain range and the Dasht-e Kavir and Dasht-e Lut desert basins, result in a somewhat isolated location likely restricting gene-flow towards the west and east already in historic times. In fact, F_{st} values between the north Iranian population in Touran and the south Iran population in Bahram-e-Goor were much larger than between Touran and the autochthonous populations in nearby Badhyz and the distant Mongolian Gobi. Still, F_{st} values place the population in Touran closest to Bahram-e-Goor and structure analysis suggests the population in Touran to be at the hybridization zone between the south Iranian ("blue") and the Turkmen ("orange/yellow") cluster. These results are in accordance with the fact the wild ass once had a continuous range in the region all the way from Mongolia into southern Iran (this also explains the significant contribution of the Mongolian "grey" cluster to the population in Badkyz and Touran).

Species and subspecies status of Asiatic wild asses have been subject to change and remain disputed, and the genetic similarity and historic co-occurrence of different haplotypes in the Asiatic wild ass group, may not justify subdivision into species or subspecies (Bennett et al. 2017). Past studies have not specifically taken population location into account or subsequently lumped results based on national borders. The inclusion of the additional haplotypes from this study and reanalysis with previously published Asiatic wild asses is still inconclusive and neither supports nor rejects an independent evolutionary past between the southern Iranian and Turkmen wild ass population. The overall picture may be compromised by small samples sizes, the opportunistic nature of past and

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present sampling and/or the special characteristics of the D-loop; the use of which in the context of human evolution has been questioned (Ingman et al. 2000).

Although there remains uncertainty over the evolutionary past of the different Asiatic wild ass groups, we feel there is enough evidence of a genetic sub-structure. Following a precautionary principle, we hence suggest subdividing Asiatic wild asses from Central Asia and Mongolia into three Management Units (MUs; Funk et al. 2012): 1) a Turkmen kulan MU comprising of animals originating from Turkmenistan (from Badhyz and its derivative reintroduced populations), 2) a Persian onager MU comprising of animals from Bahram-e-Goor, and 3) a Gobi khulan MU comprising of animals from southern Mongolia and northern China (Fig. 2; also see Bennett et al. 2017). The animals from Touran in northern Iran should be considered remnants of the former contact zone between the Turkmen kulan and Persian onager and a reminder of the long-term goal to reconnect the now isolated MUs.

However, when it comes to captive breeding, population supplementation, or reintroduction we recommend following a genetic cluster recognition approach to maintain the remaining genetic variability as well as potential local adaptations in the current MU cores (Hausknecht et al. 2014). Reintroductions should use source animals most similar to the closest MU. There is also a need to recognize that ESUs do not correspond with national borders (e.g. the largest population of Turkmen kulan is now in Altyn Emel, Kazakhstan and the population in Touran in northern Iran consists of individuals falling into two different MUs) and hence long term conservation will need regional trans-boundary population level management plans; a recommendation also in line with the Central Asian Mammals Initiative (CAMI) of the Convention of Migratory Species (CMS; http://www.cms.int/cami/). However, given the precarious state of Asiatic wild asses in Central Asia, the highest conservation priority should be given to recovering and safeguarding the three remaining autochthonous populations of Asiatic wild ass in the region, these being Bahram-e-Goor and Touran in Iran, and Badhyz in Turkmenistan; although for the latter it may already be too late.

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Population level genetic characterization and management recommendations

The autochthonous population in Bahram-e-Goor was most distinct. Reduced allelic richness and slightly elevated inbreeding coefficients are a reminder of the recent population low but are currently little reason for concern as the population is increasing. Management should aim towards supporting the increase in both numbers and range to increase demographic resilience and avoid future loss of genetic diversity.

The autochthonous population in Touran was quite diverse and the inbreeding coefficient low, suggesting that the Touran population may be larger than currently assumed. Population estimates from 2016 suggest that numbers are increasing (B. Shahriari, DoE, pers. comm. 2017). Given the small number of samples from this area, the Touran population likely contains an even higher genetic variability. Reintroduction initiatives aiming at reestablishing Asiatic wild ass in northern Iran (e.g. in Kavir or Kosh Yeilagh protected areas) should use stock derived from Touran. Ideally captive breeding initiatives should not mix animals from Touran and Bahram-e-Goor, but rather keep separate breeding programs.

The inbreeding coefficient of the captive Gourab population was extremely high and in combination with the poor reproductive output in recent years confirms inbreeding depression. In 2014, the DoE successfully captured and transferred six wild asses (two stallions, four mares) from Bahram-e-Goor to different captive facilities to increase the genetic basis of their captive breeding program. Three of these animals were sent to Gourab (B. Shahriari, pers. comm. 2014) and hence this captive population, which is rather close to Bahram-e-Goor, now constitutes of a mix of animals from Bahram-e-Goor and Touran. Unfortunately, all remaining animals at Gourab recently escaped into the wild.

The autochthonous population in Badhyz, showed a relative low level of observed heterozygosity, a high inbreeding coefficient, and a high proportion of inbred individuals which together with recent field surveys all points towards a rapidly declining population at the brink of extinction (Kaczensky and Linnell 2015). Judging from the genetic variability in the reintroduced populations in Altyn Emel and

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Gury Howdan and population genetics data from other equid populations (Table S1), it appears that the first bottleneck in the 1940s was unlikely to have resulted in the loss of much genetic variability. However, in the 1940s there were still transboundary populations between Iran, Turkmenistan and Afghanistan, and the recovery of the Badhyz population has in part been attributed to the immigration of animals from Afghanistan (Bannikov 1981). Nowadays, wild asses are extinct in Afghanistan and the transboundary population between Turkmenistan and Iran seems extinct and hence no rescue effect via immigration can be expected. In addition, the international borders with Iran and Afghanistan are now heavily fenced and constitute absolute barriers to wild ass movements (Linnell et al. 2016). Poaching levels in Badhyz seem very high, and without immediate and concerted actions to stop poaching the prospects for this population are dire, regardless of its genetic potential (Kaczensky and Linnell 2015).

The high inbreeding coefficient in the Gury Howdan population seems to be primarily the consequence of few founders and a very small population size over the past 10 years. With little potential for population expansion due to human encroachment on their habitat, the Gury Howdan animals are likely to face inbreeding depression soon. Equally low levels of heterozygosity have only been reported for Cape Mountain zebra (*Equus zebra*; Table S1), which have become susceptible to sarcoid tumors, likely a consequence of low genetic variability (Sasidharan et al. 2011). However, expected heterozygosity and allelic richness are still within ranges documented for captive populations of wild equids and domestic breeds (Table S1) and the small population holds allele frequencies which have become rare in the other populations and thus contributes to the overall differentiation. Ideally, the Gury Howdan animals could be returned to Badhyz once poaching has been controlled.

The low observed heterozygosity and high inbreeding coefficient in the reintroduced, but large, Altyn Emel population is a reminder of its recent past with two serial founder events followed by rapid population growth. Expected heterozygosity is only slightly lower than that in Badhyz and Touran and thus the Altyn Emel population seems to have retained most of the diversity of the autochthonous

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source population. Expected heterozygosity is still higher than in the reintroduced population in Israel and within the range of values of the still abundant Plains zebra (*Equus quagga*; (Lorenzen et al. 2008). The Altyn Emel population is presently the only population large enough to allow the removal of larger numbers of animals for reintroductions elsewhere in the region (Kaczensky et al. 2016b, Kaczensky et al. 2017). Given the current discrepancy between observed and expected heterozygosity, reintroductions initiatives using animals from Altyn Emel should transfer enough animals to increase the probability of their founders representing the full range of the remaining genetic variability with follow-up genetic monitoring to confirm this assumption.

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Table 1 Microsatellite diversity indices for kulan (*Equus hemionus*) populations of Central Asia. Sample size (*N*), status (S, r = reintroduced, a = autochthonous, c = captive) average number of alleles per locus (*A*), mean allelic richness per population (A_R), private alleles (A_R), observed (H_O) and expected (H_E) heterozygosity, individual fixation index (F_{IS}), mean individual inbreeding (F_{ind}), test of bottleneck (*M*) using the M-ratio test (Garza and Williamson 2001) and result of Hardy-Weinberg probability test for deviation from expected Hardy-Weinberg proportions (P_{HW}) with number of loci deviating from Hardy-Weinberg-Equilibrium after Bonferroni correction.

	S	***N***	***A***	A_R	A_{Priv}	H_O	H_E	F_{IS}	F_{ind}	***M***	P_{HW}
Kazakhstan											
AE (Altyn Emel)	r	67	8.9	4.3	0	0.390	0.730	0.468	0.476	0.74	9
average											
Turkmenistan											
TGH (Gury Howdan)	r	9	4.3	3.8	1	0.305	0.679	0.569	0.498	0.67	3
TB (Badhyz)	a	32	8.6	4.8	1	0.389	0.761	0.493	0.488	0.72	7
average			*6.4*	*4.3*		*0.347*	*0.720*	*0.531*	*0.493*		
Mongolia											
MGB (Gobi B)	a	19	9.7	5.7	2	0.776	0.843	0.081	0.197	0.73	1
MGA (Gobi A)	a	18	8.6	5.4	3	0.690	0.828	0.171	0.239	0.74	1
MGS (Small Gobi)	a	43	10.7	5.3	1	0.740	0.823	0.102	0.228	0.74	2
Average			*9.63*	*5.50*		*0.735*	*0.831*	*0.118*	*0.221*		
Iran											
IB (Bahram-e-Goor)	a	16	4.6	3.5	1	0.479	0.598	0.204	0.281	0.71	1
IG (Gourab)	c	8	3.7	3.4	1	0.177	0.651	0.745	0.597	0.62	4
IT (Touran)	a	6	4.8	4.5	2	0.696	0.769	0.102	0.204	0.71	1
average			*4.3*	*3.8*		*0.451*	*0.672*	*0.350*	*0.361*		

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Table 2 Pairwise estimates of F_{ST} values (top diagonal) and Jost's measure (Jost 2008; left diagonal) of estimated genetic differentiation (D_{EST}). Bold = values between autochthonous populations

	AE	TGH	**TB**	**MGB**	**MGA**	**MGS**	**IB**	IG	**IT**
AE		0.1411	0.0450	0.1162	0.1266	0.1347	0.2160	0.1504	0.1087
TGH	0.2565		0.1338	0.1031	0.1258	0.1275	0.2605	0.1915	0.1485
TB	0.1018	0.3152		**0.0843**	**0.0853**	**0.1009**	**0.1845**	0.1212	**0.0887**
MGB	0.3950	0.3076	**0.2443**		**0.0059**	**0.0173**	**0.1433**	0.1299	**0.0713**
MGA	0.4375	0.4218	**0.2937**	**0.0310**		**0.0068**	**0.1369**	0.1103	**0.0633**
MGS	0.4698	0.4015	**0.3200**	**0.0463**	**0.0093**		**0.1611**	0.1351	**0.0774**
IB	0.5389	0.5341	**0.4646**	**0.3960**	**0.3813**	**0.4597**		0.1667	**0.1248**
IG	0.3623	0.3609	0.3589	0.4868	0.3507	0.4369	0.2840		0.0505
IT	0.2737	0.3457	**0.2050**	**0.3099**	**0.2218**	**0.2716**	**0.2090**	0.1546	

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744 **Fig. 1** Autochthonous and re-introduced Asiatic wild ass populations in Central Asia. Analysed
745 populations are shown with thick borders and the text boxes give the number of founders of the two
746 reintroduced and the captive population analysed.

747 **Fig. 2** Genetic characterization of nine wild ass (sub)populations in Mongolia and Central Asia:
748 A) Geographic location, genetic constitution (color), and observed heterozygosity (size), B)
749 Clustering of individuals from each population (all depicted in the mean population color) based
750 on Discriminant Analysis of Principal Components (DAPC), and C) individual genetic
751 characterization by population. The colour of the dots corresponds to the result of the DAPC
752 (Jombart et al. 2010). The first three DA eigenvalues show 42%, 23%, and 13% of the retained
753 variation. The similarity of the dots represents the genetic similarity of populations (A, and B) or
754 individuals (C). The side length of the square in A is proportional to the square root of observed
755 heterozygosity H_O of the corresponding population. Arrows connect source populations with
756 their derivative reintroduced populations.

757 **Fig. 3** Individual inbreeding coefficient (F_{ind}) by population.

758 **Fig. 4** Structure analysis (no admixture) assignment of individual samples to main genetic
759 clusters (K=2, K=3, and K=4). The histograms are showing the cluster distribution per individual,
760 the pie char per population (K=4).

761 **Fig. 5** Maximum likelihood (ML) estimates of phylogenetic relationships of Gobi khulan, Kiang,
762 Persian onager and Turkmen kulan HVRI sequences using T92+G, Tamura 3-parameter, Tamura
763 (1992) as the best fit model based on the Bayesian Information Criterion approach. Nodes are
764 labeled with the highest bootstrap support by ML (1.000 replications). With triangle: Sequences of
765 this study.

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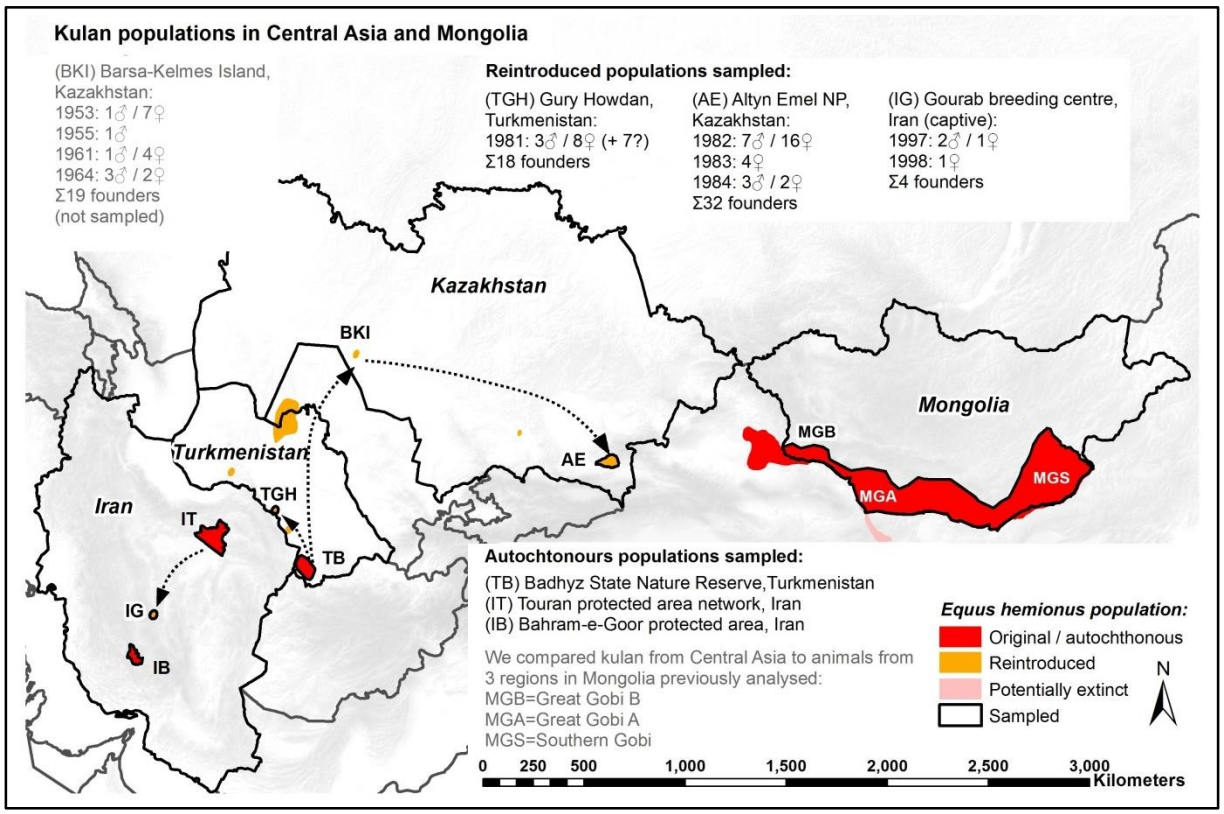


Fig 1

Kaczensky, Petra; Kovtun, Ekaterina; Habibrakhmanov, Rustam; Hemami, Mahmoud-Reza; Linnell, John D.C.; Khaleghi, Amirhossein; Linnell, John Durrus; Rustamov, Eldar; Sklyarenko, Sergey; Walzer, Chris; Zuther, Steffen; Kuehn, Ralph. Genetic characterization of free-ranging Asiatic wild ass in Central Asia as a basis for future conservation strategies. Conservation Genetics 2018 DOI:10.1007/s10592-018-1086-3

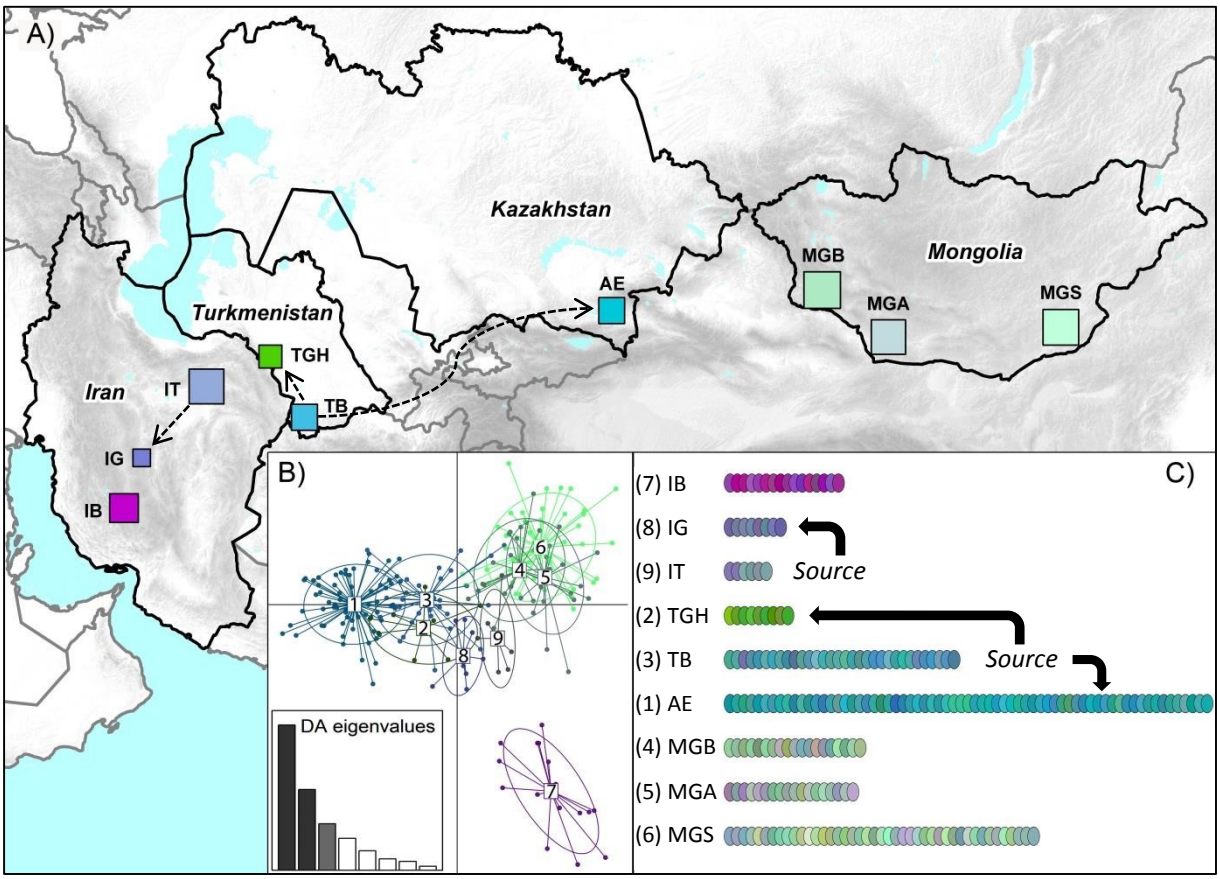
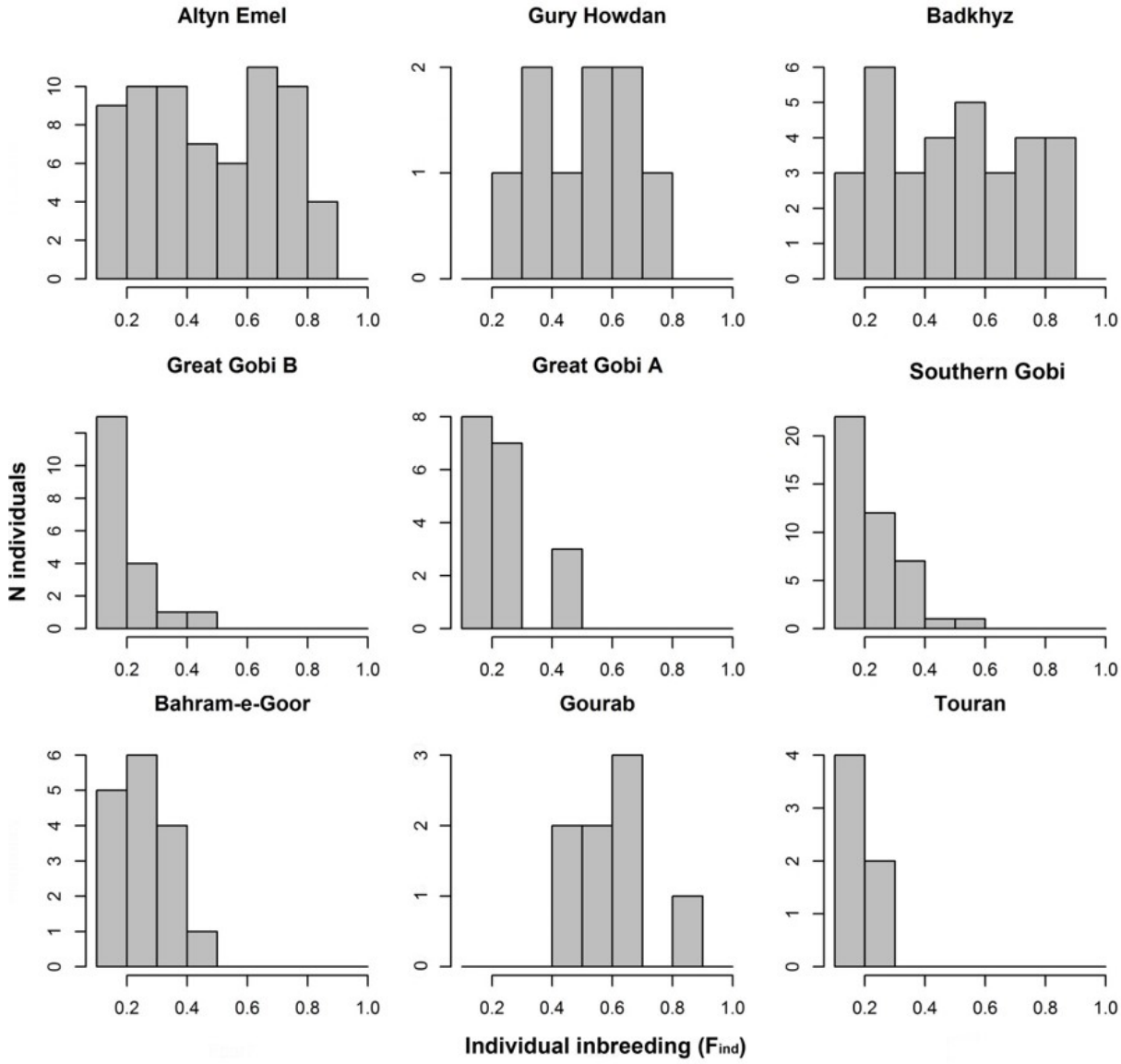


Fig. 2

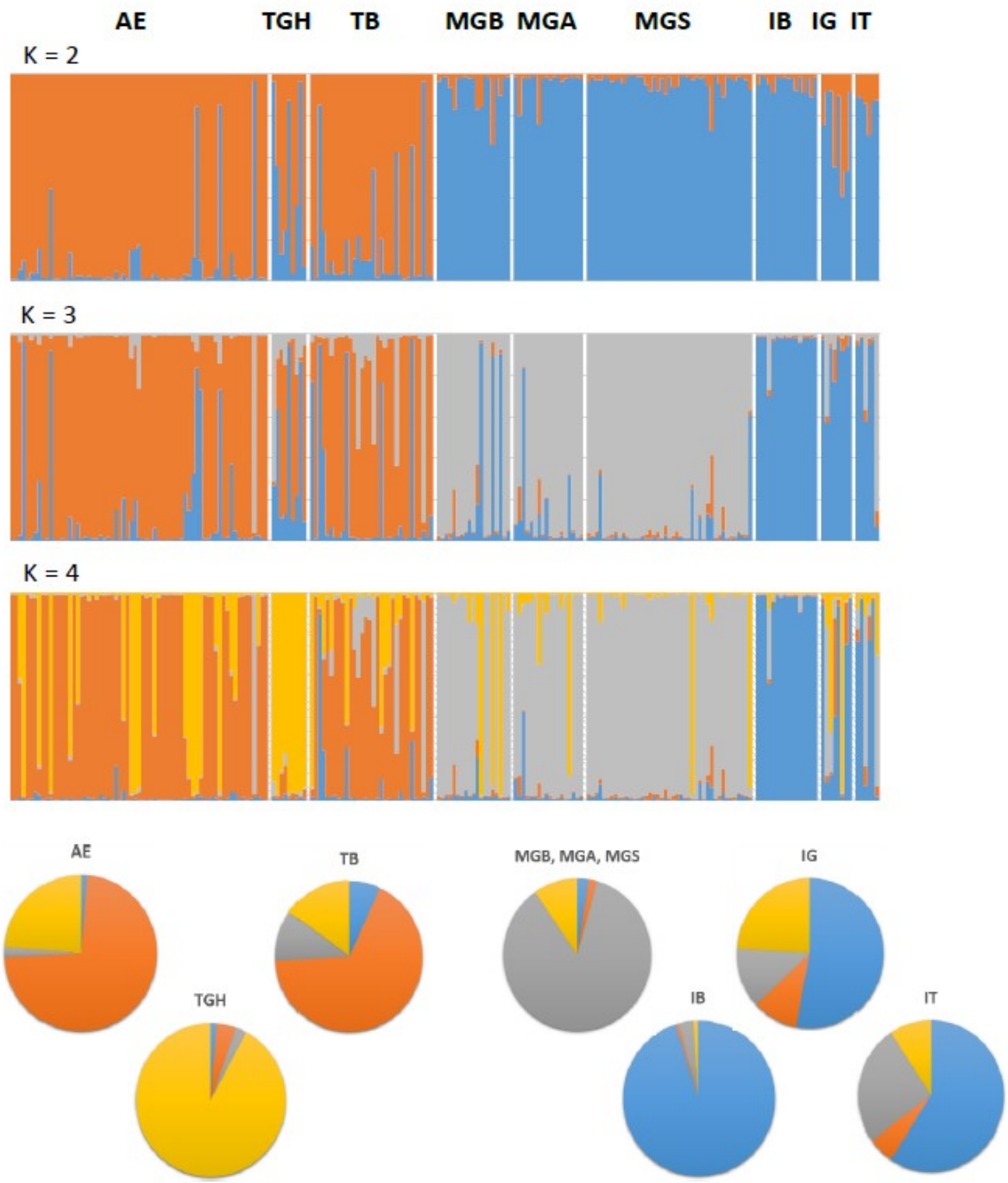
Kaczensky, Petra; Kovtun, Ekaterina; Habibrakhmanov, Rustam; Hemami, Mahmoud-Reza; Linnell, John D.C.; Khaleghi, Amirhossein; Linnell, John Durrus; Rustamov, Eldar; Sklyarenko, Sergey; Walzer, Chris; Zuther, Steffen; Kuehn, Ralph. Genetic characterization of free-ranging Asiatic wild ass in Central Asia as a basis for future conservation strategies. Conservation Genetics 2018 DOI:10.1007/s10592-018-1086-3



787

788 **Fig. 3**

Kaczensky, Petra; Kovtun, Ekaterina; Habibrakhmanov, Rustam; Hemami, Mahmoud-Reza; Linnell, John D.C.; Khaleghi, Amirhossein; Linnell, John Durrus; Rustamov, Eldar; Sklyarenko, Sergey; Walzer, Chris; Zuther, Steffen; Kuehn, Ralph. Genetic characterization of free-ranging Asiatic wild ass in Central Asia as a basis for future conservation strategies. Conservation Genetics 2018 DOI:10.1007/s10592-018-1086-3



789

790 **Fig. 4**

Kaczensky, Petra; Kovtun, Ekaterina; Habibrakhmanov, Rustam; Hemami, Mahmoud-Reza; Linnell, John D.C.; Khaleghi, Amirhossein; Linnell, John Durrus; Rustamov, Eldar; Sklyarenko, Sergey; Walzer, Chris; Zuther, Steffen; Kuehn, Ralph. Genetic characterization of free-ranging Asiatic wild ass in Central Asia as a basis for future conservation strategies. Conservation Genetics 2018 DOI:10.1007/s10592-018-1086-3

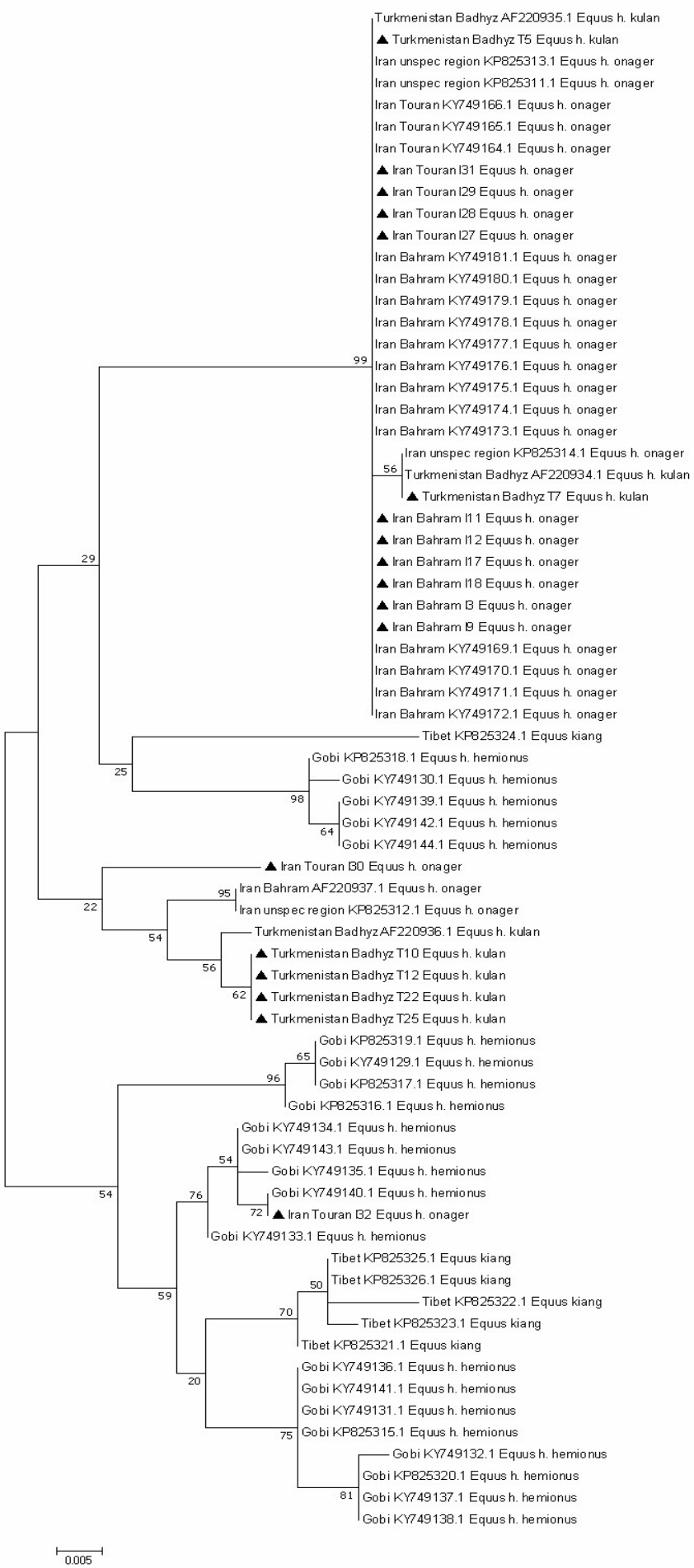


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Fig. 5

Kaczensky, Petra; Kovtun, Ekaterina; Habibrakhmanov, Rustam; Hemami, Mahmoud-Reza; Linnell, John D.C.; Khaleghi, Amirhossein; Linnell, John Durrus; Rustamov, Eldar; Sklyarenko, Sergey; Walzer, Chris; Zuther, Steffen; Kuehn, Ralph. Genetic characterization of free-ranging Asiatic wild ass in Central Asia as a basis for future conservation strategies. Conservation Genetics 2018 DOI:10.1007/s10592-018-1086-3

792 **SUPPLEMENTARY MATERIAL**

793 **Table S1** Population genetics studies using microsatellites in other equid populations: expected

794 heterozygosity (H_E), observed heterozygosity (H_O), number of alleles (A), Allelic richness (A_R).

Species	Subspecies / breed / population	N animals	N micro-satellites	Known founders	H_E	H_O	A	A_R	Reference
Domestic horse (*Equus caballus*)	Hanoverian Warmblood	47	30	NA	0.74	0.74	6.70	---	Aberle et al. 2004
	Icelandic horse	45	30	NA	0.73	0.72	6.43	---	
	Arabian	25	30	NA	0.57	0.58	4.37	---	
	Sorraia horse	23	30	NA	0.53	0.53	3.43	---	
Przewalski's horse (*Equus ferus przewalskii*)	Captive	18	30	13	0.53	0.47	3.83	---	Aberle et al. 2004
Mountain zebra (*Equus zebra*)	Hartmann's mountain zebra	196	15	autochthonous	0.54	0.48	5.55	---	Moodley & Harley 2006
	Cape mountain zebra	95	15	5-16	0.38	0.24	4.07	---	
	Captive	6	23	NA	0.37	0.31	2.75	2.47	Ito et al. 2015
Plains zebra (*Equus quagga*)	Kenya - Masai Mara	14	7	autochthonous	0.79	---	7.30	---	Lorenzen et al. 2008
	Tanzania - Maswa	11	7	autochthonous	0.74	---	5.90	---	
	Tanzania - Burko	12	7	autochthonous	0.71	---	6.70	---	
	Tanzania - Ikiri-Rungwa	15	7	autochthonous	0.80	---	7.10	---	
	Zambia - Lochnivar South	10	7	autochthonous	0.75	---	5.70	---	
	Namibia - Etosha	18	7	autochthonous	0.80	---	8.60	---	
	Captive	27	23	NA	0.57	0.50	5.32	3.21	Ito et al. 2015
Grevy's zebra (*Equus grevyi*)	Captive	52	25	NA	0.43	0.40	4.07	2.44	Ito et al. 2015
Asiatic wild ass (*Equus hemionus*)	Persian onager - captive	60	12	NA	0.60	0.51	4.50	5.33	Nielsen et al. 2007
	Persian onager - Bahram-e-Goor (IB)	16	9	autochthonous	0.60	0.48	4.56	3.52	this study
	Persian onager - Gourab captive (IG)	8	9	4-5	0.65	0.18	3.67	3.41	
	Persian onager – Touran (IT)	6	9	autochthonous	0.77	0.70	4.78	4.55	
	Turkmen kulan – Badhyz (TB)	32	9	autochthonous	0.76	0.39	8.56	4.81	
	Turkmen kulan - Gury Howdan (TG)	9	9	18	0.68	0.31	4.33	3.79	
	Turkmen kulan - Altyn Emyl (AE)	67	9	32	0.73	0.40	8.89	4.28	
	Gobi khulan - Great Gobi B	19	9	autochthonous	0.84	0.78	9.67	5.73	Kaczensky et al. 2011
	Gobi khulan - Great Gobi A	18	9	autochthonous	0.83	0.69	8.56	5.43	
	Gobi khulan - SE Gobi	43	9	autochthonous	0.82	0.74	10.67	5.34	
	Persion onager x Turkmen kulan - breeding core	27.9	8	11	0.56	0.58	3.60	---	Renan et al. 2015
	Persion onager x Turkmen kulan - wild	114.8	8	11	0.54	0.56	3.40	---	
Domestic donkey (*Equus arsinus*)	Northeast Africa	60	15	NA	0.63	0.58	---	5.81	Rosenbom et al. 2015
	Near East	20	15	NA	0.66	0.56	---	5.67	
	Arabian Peninsula	49	15	NA	0.66	0.61	---	5.93	
African wild ass *(Equus africanus*)	Wildlife reserves & zoos	22	15	NA	0.59	---	5.06	---	Rosenbom et al. 2012

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Table S2 Summary for the nine polymorphic microsatellite loci: Allelic richness (A_R), number of private alleles (A_P), expected heterozygosity (H_E), calculated F_{IS} values, and departures from Hardy–Weinberg equilibrium (*** = P < 0.0055). Autochtonous: TB = Badhyz, Turkmenistan; IB = Bahram-e-Goor and IT = Touran, Iran; MGB = Great Gobi B, MGA = Great Gobi A, and MGS = southern Gobi, Mongolia; *Reintroduced: AE = Altyn Emel, Kazakhstan; TGH = Gury Howdan, Turkmenistan; **Captive: IG = Gourab breeding center, Iran.

	AE*	TGH*	TB	MGB	MGA	MGS	IB	IG**	IT
ASB23									
A_R	5.8	3.5	5.9	7.2	7.2	6.7	3.0	5.3	5.5
H_E	0.866	0.500	0.853	0.923	0.918	0.900	0.688	0.911	0.867
F_{IS}	0.466	0.556	0.471	0.030	-0.089	-0.008	0.455	0.863	0.038
HW	***		***					***	
COR007									
A_R	3.5	2.8	3.1	4.6	4.0	4.4	3.8	3.0	3.7
H_E	0.550	0.583	0.489	0.744	0.724	0.758	0.679	0.732	0.583
F_{IS}	0.592	0.429	0.181	0.081	0.309	0.157	0.448	0.488	0.429
HW	***					***	***		
COR58									
A_R	3.7	3.6	5.9	6.7	4.9	6.0	4.5	4.0	4.0
H_E	0.632	0.681	0.852	0.899	0.838	0.871	0.781	0.900	0.800
F_{IS}	0.294	0.673	0.684	0.005	0.403	0.180	0.200	1.000	0.375
HW	***	***	***						
COR70									
A_R	4.7	4.6	5.7	6.2	7.0	5.583	2.920	2.000	4.667
H_E	0.782	0.881	0.837	0.885	0.914	0.838	0.502	0.600	0.867
F_{IS}	0.533	0.838	0.629	0.048	0.052	0.056	0.253	1.000	0.615
HW	***	***	***						
SGCV28									
A_R	4.1	4.8	4.6	6.1	5.4	5.1	4.8	3.6	7.1
H_E	0.752	0.778	0.809	0.886	0.852	0.819	0.815	0.705	0.917
F_{IS}	0.455	0.143	0.442	0.185	0.193	0.092	0.079	0.291	-0.091
HW	***		***						
COR18									
A_R	4.4	4.0	4.9	4.2	5.0	5.3	2.0	2.4	3.0
H_E	0.787	0.775	0.806	0.734	0.788	0.802	0.233	0.274	0.575
F_{IS}	0.417	0.484	0.587	0.211	0.506	0.101	0.196	-0.043	-0.391
HW	***		***		***				
COR71									

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A_R	4.7	4.3	4.9	6.1	6.2	6.2	3.9	4.5	5.0
H_E	0.768	0.806	0.818	0.874	0.892	0.875	0.721	0.786	0.825
F_{IS}	0.545	0.724	0.369	0.097	0.142	0.229	0.133	0.818	-0.212
HW	***	***	***					***	***
LEX68									
A_R	4.2	2.7	4.9	5.3	4.7	4.3	5.0	3.0	5.0
H_E	0.749	0.607	0.824	0.832	0.814	0.767	0.821	0.667	0.875
F_{IS}	0.532	0.529	0.636	-0.076	-0.024	0.037	-0.142	0.750	0.086
HW	***		***	***					
UM11									
A_R	3.5	3.7	3.1	5.2	4.5	4.5	1.7	2.9	3.0
H_E	0.711	0.759	0.620	0.827	0.753	0.786	0.179	0.679	0.667
F_{IS}	0.372	0.671	0.245	0.173	0.115	0.069	0.651	1.000	0.000
HW	***							***	

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Table S3 Pairwise straight-line distances (in km) among the nine Asiatic wild ass populations. Autochthonous: TB = Badhyz, Turkmenistan; IB = Bahram-e-Goor and IT = Touran, Iran; MGB = Great Gobi B, MGA = Great Gobi A, and MGS = southern Gobi, Mongolia; *Reintroduced: AE = Altyn Emel, Kazakhstan; TGH = Gury Howdan, Turkmenistan; **Captive: IG = Gourab breeding center, Iran.

	AE*	TGH*	TB	MGB	MGA	MGS	IB	IG**	IT
AE*									
TGH*	1780								
TB	1600	320							
MGB	1000	2680	2580						
MGA	1320	2970	2840	360					
MGS	2170	3820	3690	1560	850				
IB	2550	1000	950	3510	3760	4600			
IG**	2380	770	780	3560	3620	4470	270		
IT	2000	330	480	3000	3280	4130	710	450	

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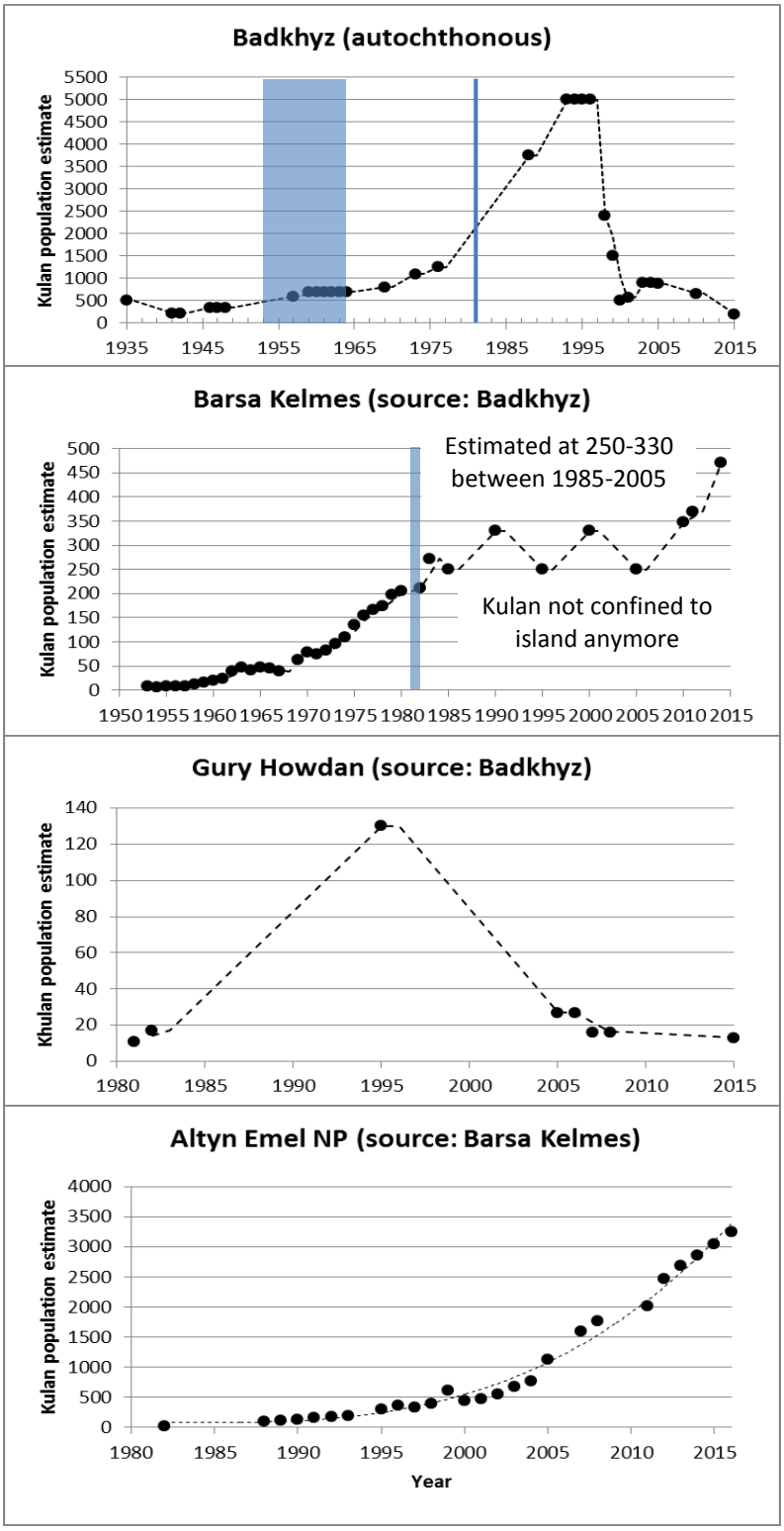

831

832 **Fig. S1a** Population history of the source and reintroduced populations of Turkmen kulan
833 sampled in Turkmenistan and Kazakhstan 2014-2015.Dashed lines show the moving average or a
834 polynomal fit to the available population estimates. Blue bars depict periods when kulan were
835 captured for reintroductions to Barsa Kelmes, Gury Howdan, or Altyn Emel (also see Fig. 1).

Kaczensky, Petra; Kovtun, Ekaterina; Habibrakhmanov, Rustam; Hemami, Mahmoud-Reza; Linnell, John D.C.; Khaleghi, Amirhossein; Linnell, John Durrus; Rustamov, Eldar; Sklyarenko, Sergey; Walzer, Chris; Zuther, Steffen; Kuehn, Ralph. Genetic characterization of free-ranging Asiatic wild ass in Central Asia as a basis for future conservation strategies. Conservation Genetics 2018 DOI:10.1007/s10592-018-1086-3

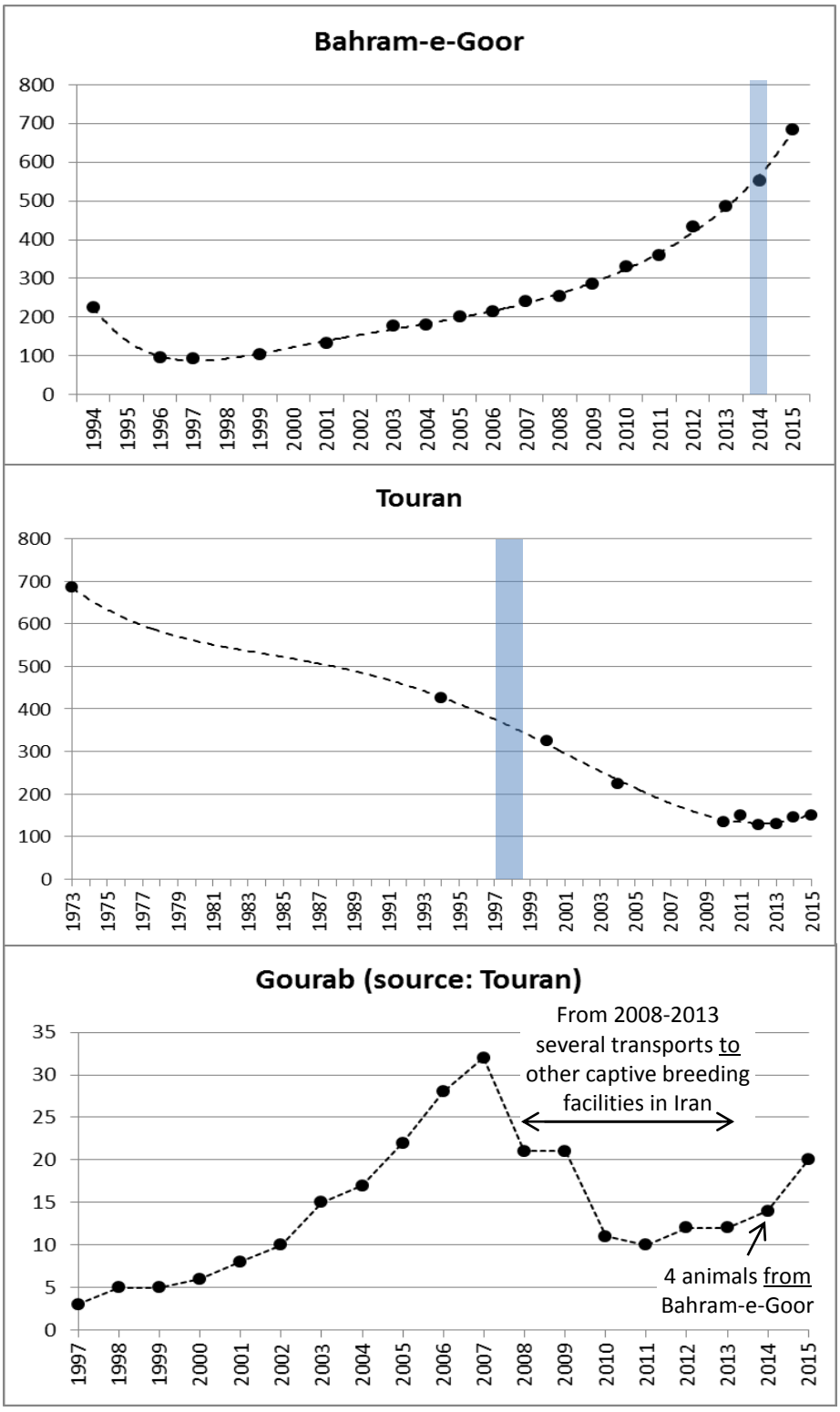

Figure S1b: Population history of the two remaining autochtonous populations of Persian onager in Bahram-e-Goor and Touran, and the small captive population in Gourab founded with animals from Touran. Dashed lines show the connection, moving average or a polynomal fit to the available population estimates.The blue bars depict population removals for captive breeding facility in Gourab.

Kaczensky, Petra; Kovtun, Ekaterina; Habibrakhmanov, Rustam; Hemami, Mahmoud-Reza; Linnell, John D.C.; Khaleghi, Amirhossein; Linnell, John Durrus; Rustamov, Eldar; Sklyarenko, Sergey; Walzer, Chris; Zuther, Steffen; Kuehn, Ralph. Genetic characterization of free-ranging Asiatic wild ass in Central Asia as a basis for future conservation strategies. Conservation Genetics 2018 DOI:10.1007/s10592-018-1086-3

Nature of population estimates

Population development reconstruction is based on the published and unpublished sources listed below. Estimates should be treated as the best available information as methods were neither standardized within areas over time, nor among areas. Population estimates include extrapolations from counts at focal points, aerial or ground surveys of parts or the entire kulan range and attempted total counts. Recent estimates in Turkmenistan, Kazakhstan, and Iran are primarily based on total counts of simultaneously operating counting teams or expert assessments based on signs. In Bahram-e-Goor total counts in 2009 were in the same magnitude as estimates derived from DISTANCE sampling estimates (Hemami and Momeni, 2013).

Estimates given in different publications, reports or protected area statistics vary somewhat between sources and time periods. We tried to compile the data from the longest time series available at one place and/or the most likely estimates. In general, differences are small and do not affect the overall magnitude or trends in kulan populations.

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B. Shahriari, DoE Isfahan, for Gourab breeding center, Iran

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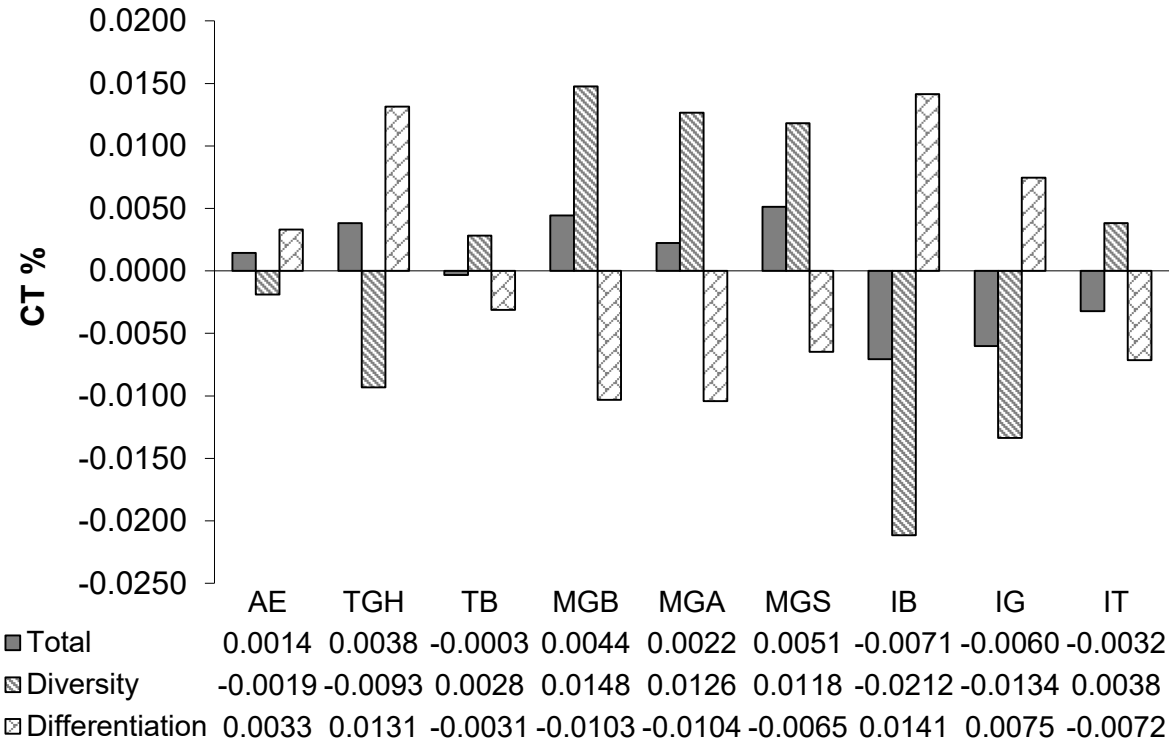
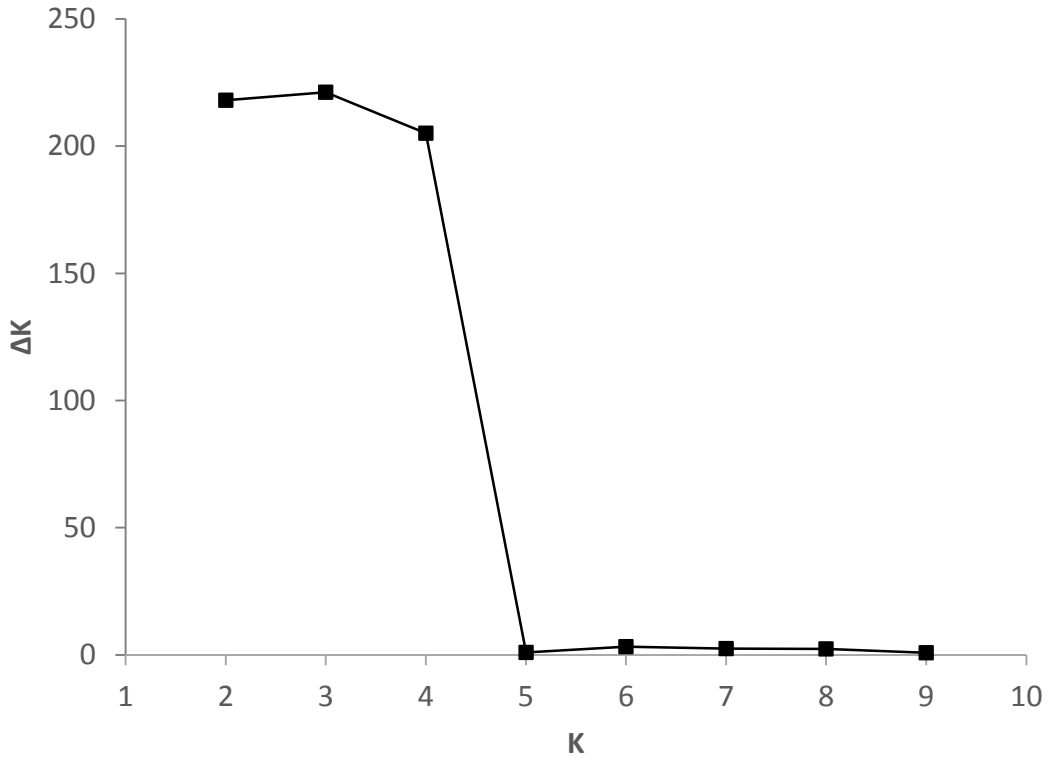


Fig. S2 Heterozygosity contribution CT to total diversity (subdivided into a diversity and a differentiation compound) for 9 Central Asia Kulan (*Equus hemionus*) populations based on CONTRIB-calculations according to Petit et al. (1998).

Kaczensky, Petra; Kovtun, Ekaterina; Habibrakhmanov, Rustam; Hemami, Mahmoud-Reza; Linnell, John D.C.; Khaleghi, Amirhossein; Linnell, John Durrus; Rustamov, Eldar; Sklyarenko, Sergey; Walzer, Chris; Zuther, Steffen; Kuehn, Ralph. Genetic characterization of free-ranging Asiatic wild ass in Central Asia as a basis for future conservation strategies. Conservation Genetics 2018 DOI:10.1007/s10592-018-1086-3

912 **Figure S3** ΔK values in relation to the number of clusters (K) of 218 Asiatic wild ass samples
913 from 9 locations in Central Asia and Mongolia.



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