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Genetic diversity of the endangered Mongolian saiga antelope *Saiga tatarica mongolica* (Artiodactyla: Bovidae) provides insights into conservation

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The Saiga antelope (*Saiga tatarica*) is one of the few megafauna species from the mammoth steppe still living today. Currently, saiga are classified as critically endangered, persisting only in small areas of Central Asian steppe and desert ecosystems. The species is divided into two subspecies: *Saiga tatarica mongolica* and *Saiga tatarica tatarica*. In this study, we have for the first time characterized the genetic diversity of the Mongolian saiga (*S. t. mongolica*) using both mitochondrial DNA and microsatellite markers. We also analysed *S. t. tatarica* specimens in order to genetically compare both subspecies. The mitochondrial control region was sequenced for a total of 89 individuals: 20 skin, 53 umbilical cord, three placentae and a muscle sample from *S. t. mongolica*, and a total of 12 hair samples from *S. t. tatarica*. Additionally, 19 microsatellites developed for saiga antelope were also screened. Our results revealed that the Mongolian saiga presents very low genetic diversity at the mitochondrial level, with no shared mitochondrial haplotype between the two subspecies. Low genetic diversity is also present at the autosomal level, with most loci having low heterozygosity (Ho/He) and a low number of alleles per locus. Despite the low genetic diversity, we found no separation between the subpopulations in Mongolia, indicating that conservation corridors are actually promoting contact between different herds. Our results validate current conservation efforts and inform the implementation of new measures to increase the viability of the *S. t. mongolica* subspecies.

ADDITIONAL KEYWORDS: Central Asian Steppe – conservation – DNA – Mongolia – saiga antelope.

INTRODUCTION

The saiga antelope *Saiga tatarica* (Linnaeus, 1766) is one of the few species from the mammoth steppe megafauna still living today. It is a small nomadic, gregarious antelope perfectly adapted to the arid landscape of the Central Asian plains. During the last interglacial, the range of the species spanned all the way from France to the Northwest Territories of Canada; nevertheless, by the end of the Pleistocene, it was restricted to the steppes of Central Asia (Campos *et al.*, 2010; Jürgensen *et al.*, 2017). Historical records

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indicate that it remained extremely abundant until the end of the Soviet Union (1991), after which saiga populations were reduced by over 95% (Milner-Gulland *et al.*, 2001).

Nowadays, saiga antelope is represented by two recognized subspecies: *Saiga tatarica tatarica* (*S. t. tatarica*) and *Saiga tatarica mongolica* (*S. t. mongolica*) Bannikov, 1946, which are defined based on morphological, ecological, and geographical differences (Bekenov *et al.*, 1998); although some authors, due to these factors, consider them two distinct species (Carroll, 1988; Wilson & Reeder, 2005). The Mongolian saiga is sometimes also referred to as *Saiga borealis mongolica* (Wilson & Reeder, 2005). *Saiga tatarica tatarica* is present in countries in the pre-Caspian region including Kazakhstan, Russia, Uzbekistan and Turkmenistan, and *S. t. mongolica* inhabits in the Great Lakes depression in western Mongolia (Fig. 1A). At present *S. t. mongolica* and *S. t. tatarica* are isolated by the Altai Mountain Range, with approximately 1500 km separating them (IUCN, 2018). Current available genetic studies based on the mitochondrial control region (CR) support the split into two subspecies (Kholodova *et al.*, 2006).

The species is nowadays listed on the IUCN Red List as Critically Endangered (IUCN, 2018). It also appears on Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and the Convention on the Conservation of Migratory Species of Wild Animals (CMS). The Mongolian subspecies is categorized as regionally Endangered, due to small but stable

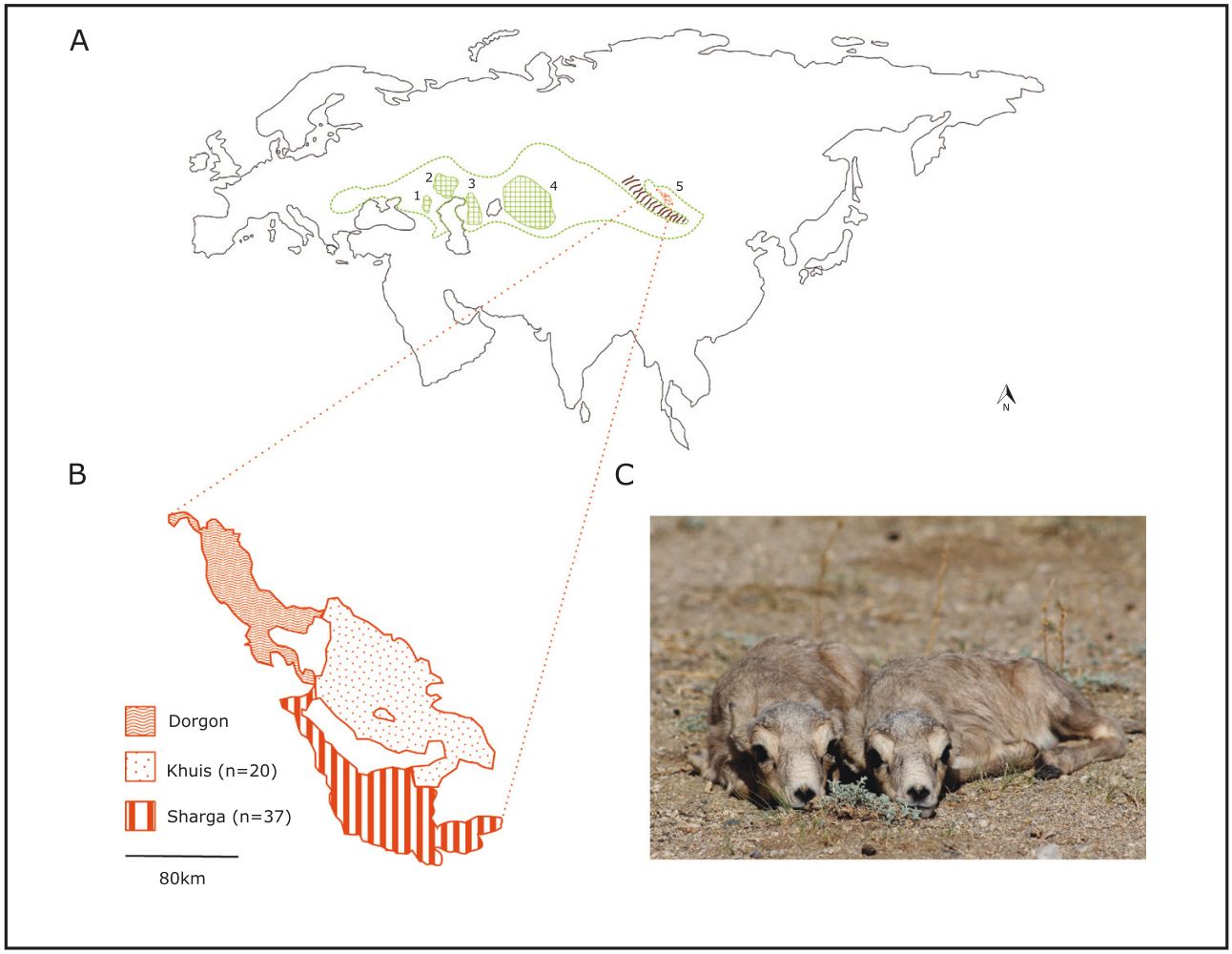

Figure 1. A, current and historical *S. tatarica* distribution range; numbers represent extant populations: (1) Kalmykia, (2) Ural, (3) Ustiurt, (4) Betpak Dala, Kazakhstan, (5) Mongolia. Dashed line represents the area where the species is known to have existed during historical times (17th–18th century). Rugged lines represent the Altai Mountains (drawn in Inkscape v.1.0.1, https://inkscape.org). B, Mongolian saiga distribution range in western Mongolia: Sharga Gobi, Khuis Gobi and Dorgon, covering an area of approximately 20 000 km^2. C, Mongolian saiga twin calves.

number of individuals (Clark *et al.*, 2006). At present, the Mongolian saiga occupies only about 20% of its former range and the majority of which, around 90%, occurs in three main regions: Sharga Gobi, Khuis Gobi and Dorgon Steppe in western Mongolia, covering an area of approximately 2000 km^2 (Fig. 1B). Additionally, there is an isolated and small population (~ 200 individuals) in and around the Mankhan Nature Reserve (Dulamtseren & Amgalan, 1995). In 1993, two nature reserves were established, the Sharga Gobi Nature Reserve (286 900 ha) and the Mankhan Nature Reserve (30 000 ha), that include portions of semi-desert and foothill areas of the Altai Mountain Range and the Darwiyn-nuru Ridge (Lushchekina *et al.*, 1999). The total potential range for the Mongolian subspecies is 13 000 km^2 (Clark *et al.*, 2006).

Saiga tatarica tatarica is a nomadic animal with wide range territories, undertaking long-distance movements between the winter and summer ranges; as opposed to *S. t. mongolica*, which demonstrates less migratory behaviour with no clearly defined summer and winter ranges (Berger *et al.*, 2008). Saiga populations are specially threatened by various anthropogenic factors, such as human pressures on the original habitats, construction of irrigation channels, obstacles preventing natural dispersion and migration, poaching, degradation of pastures, and use of water resources by grazing livestock (Bekenov *et al.*, 1998). The latter is very prominent in Mongolia where livestock husbandry is the most important local industry (Berger *et al.*, 2013), suggesting it could largely impact saiga populations, as it does for Mongolian gazelles (*Procapra gutturosa*) and argali sheep (*Ovis ammon*) in the Gobi region (Yoshihara *et al.*, 2008; Sugimoto *et al.*, 2018).

Selective poaching of saiga males for horns, used in traditional Chinese medicine, led to a population decline and resulted in skewed sex ratios, the percentage of males in an unhunted population range from 25 to 30% while in such cases it dropped to less than 10%, leading to a reproductive collapse (Bekenov *et al.*, 1998; Clark *et al.*, 2006; Cui *et al.*, 2017).

Although hunting is prohibited in all of its range, the mortality of saiga due to poaching still exists, with e.g. 4470 smuggled horns worth US$ 22 million confiscated in September 2013 in north-west China (Saiga Conservation Alliance, 2013). Saiga populations have also been affected by several recent mass die-offs caused by *Pasteurella multocida* bacteria, resulting in a precipitous decline in population sizes (Kock *et al.*, 2018; Fereidouni *et al.*, 2019; Pruvot *et al.*, 2020). Due to the combination of anthropogenic and natural factors, *S. t. tatarica* populations have had a dramatic decline, with the global population in January 2018 estimated at 159 600–160 600 (IUCN, 2018). Similarly, the Mongolian saiga has experienced dramatic population fluctuations in the past two decades, ranging between 750 and 15 000 individuals, primarily owing to stochastic events such as harsh winters and disease outbreaks, with the current population estimated at 6400 individuals. This number was severely impacted by the recent (2017) outbreak of Peste des Petits Ruminants (PPR) and the climatic conditions of the recent past (Chimeddorj *et al.*, 2019). The extremely low population sizes, especially in Mongolia, render them highly susceptible to the stochastic events that could lead to mass mortality and potentially drive this species to extinction. Small, isolated populations, such as these, are considered to be highly susceptible to both stochastic demographic risks and genetic factors, genetic drift and inbreeding, that can reduce fitness and compromise long-term persistence. Genetic drift can also lead to a loss of genetic variation diminishing the adaptive potential of a species (Kohn *et al.*, 2006). Reduced diversity may be associated with susceptibility to pathogens, which if taken to extremes could potentially result in species declines and ultimately extinction. Major histocompatibility complex (MHC) genes (class I and class II) are highly polymorphic and are among the most diverse genes in the mammalian genome. This high polymorphism is thought to be driven by host-parasite co-evolution (e.g. Spurgin & Richardson, 2010). Diversity of MHC genes is crucial to host defence against pathogens. In some studies variation in MHC diversity has been shown to correlate with factors such as parasite load and resistance to infection (e.g. Radwan *et al.*, 2014). The saiga's low genetic diversity has the risk of increasing its susceptibility to pathogens, increasing its extinction risk.

In this study, we have genetically characterized the Mongolian saiga subspecies using both mitochondrial [control region (CR)] and nuclear markers (19 microsatellites).

Given their recent population history with several boom and crash episodes, and results from previous studies based on mitochondrial DNA (Kholodova *et al.*, 2006; Sod-Erdene *et al.*, 2017), we expect to find low diversity levels. Our research will, for the first time, shed some light on the dynamics of this subspecies and provide essential information to be incorporated into the conservation of the remaining populations of this iconic steppes mammal already going on in Mongolia.

MATERIAL AND METHODS

Sample collection

We collected 77 samples in seven different field seasons: 37 umbilical cord samples collected between 2009–2012, 20 skin samples collected in 2014, and 20 samples collected in 2015 (sample details in

Supporting Information, Table S1, six samples were collected from putative twins, Fig. 1C). Saiga antelope females give birth away from the herd, in suitable birthing places, leaving the calf concealed in the steppes and returning regularly to feed it (Buuveibaatar *et al.*, 2014). All females birth their calves within a week of each other. Shortly after the peak of births, the females and newborns re-join the males. After 3–4 months, the juveniles are completely independent. All these factors make it possible for saiga populations to rapidly expand over relative short time periods, and facilitate dry umbilical cord tissue sampling, as we specifically targeted newborns when mothers were away feeding. Sampling was performed with the minimum possible disturbance and handling, covering the animal's eyes to reduce stress (Buuveibaatar *et al.*, 2013a). All the samples were collected in two main regions, the Sharga Gobi and Khuis Gobi, we were not able to collect any samples from Dorgon Steppe (see Fig. 1B). Twenty samples (STM 1-11, STM 16-22, STM 25-26) were obtained from confiscated saiga horns, which means their specific origin is unknown. Additionally, we used 12 hair *S. t. tatarica* samples, five from Betpak-Dala, Kazakhstan, and seven from Kalmykia, Russia, for comparison (see Supporting Information, Table S1).

DNA extraction and mtDNA sequencing

Genomic DNA was extracted from skin, muscle, placenta and umbilical cord samples using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, with the following modifications: tissue samples were digested in the presence of lysis buffer and proteinase K; the amount of ATL buffer, proteinase K, AL buffer and ethanol was doubled due to the high quantity of starting material. To increase DNA yield, samples were eluted in 50 µL AE and incubation time was increased to 10 min at 37 °C. Extractions were performed in batches of seven or nine samples, along with a negative control. Genomic DNA from hair samples was extracted following the protocol described in Campos & Gilbert (2012).

The complete mtDNA control region (CR) was amplified for 88 samples using the universal mammalian primers tRNAF (5' TCA ACA CCC AAA GCT GAA GT 3') and tRNAR (5' GCA TTT TCA GTG CCT TGC TT 3'). PCR amplification was performed in 25 µL, using 1 × PCR buffer, 2 mM of $MgCl_2$ (25 nM), 1.6 mg/mL Bovine Serum Albumin (BSA), 0.4 µM of each primer, 0.1 mM of dNTPs and 1 µL Ampli-Taq Gold (Life Technologies). PCR cycling conditions were 94 °C for 5 min; 40 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min, followed by 72 °C for 7 min. PCR products were visualized under UV light after electrophoresis in a 2% agarose gel. After purification PCR products were commercially sequenced (Macrogen Europe) in both directions. The sequences were edited by combining the forward and reversed strands and aligned using Geneious v.7.1.9.

Microsatellite amplification

Nineteen microsatellite markers were amplified using primers developed for *S. t. tatarica* by Nowak *et al.* (2014). PCR amplification was performed in 10 µL volumes, using 5 µL the HotStar Taq Master Mix Kit (Qiagen, Hilden, Germany), 2.5 µL H_2O, 1.5 µL sample and either 1 µL primer mix (for multiplex reactions) or 0.5 µL of each primer (for singleplex reactions). PCR cycling conditions were 95 °C for 15 min; touchdown annealing temperature: four cycles of 60 °C for 90 s, five cycles for 58 °C for 90 s, five cycles for 54 °C for 90 s, 25 cycles for 50 °C for 90 s and 72 °C for 1 min, followed by 72 °C for 30 min.

Loci were amplified using forward primers labelled with the fluorescent dyes 6-FAM, PET, NED or VIC. PCR products were separated by size on an ABI3130xl Genetic Analyzer (Applied Biosystems). Allele sizes were scored against the GeneScan 500 LIZ Size Standard, using GENEMAPPER 4.0 (Applied Biosystems) and manually checked twice. The accuracy of genotypes was confirmed through re-amplification and re-analysis of 5% of randomly selected samples for each locus, resulting in complete concordance among replicates.

To guard against contamination, all reagents used were molecular biology grade, and blank extraction and PCR controls were used.

Population genetic analyses

For the mtDNA CR sequences, the number of segregating sites (S), haplotypes (h), haplotype (Hd) and nucleotide (π) diversities were calculated using DNASP v.5 (Librado & Rozas, 2009). To visualize the relationships among the haplotypes, a median-joining network (Bandelt *et al.*, 1999) was reconstructed using PopART (Leigh & Bryant, 2015) for two data sets, one encompassing the three populations (Mongolia, Kalmykia and Kazakhstan) and another one with only the Mongolian samples. Microsatellite diversity [mean number of alleles per locus (Na), observed (Ho) and expected (He) heterozygosity for each locus/population] was evaluated, considering three starting groups corresponding to the three sampled populations—Mongolia (population 1), Kalmykia (population 2) and Kazakhstan (population 3)—using GeneAlEx 6.501 (Peakall & Smouse, 2012). The same software was used to evaluate population differentiation assessed by Fisher's analogues of the pairwise mean Fst. Inbreeding within populations

was estimated using the fixation index: Fst = (Ht–Hs)/Ht where Hs = intra population haplotype variation and Ht = total expected heterozygosity. Analyses were performed for the 19 microsatellites scored in this study (results not shown), and also for a smaller panel of 13 microsatellites that excluded four monomorphic markers (Sta02, Sta03, Sta05 and Sta69, Supporting Information, Table S2) and two markers with high levels of missing data (Sta30, monomorphic for all typed specimens, and Sta44, Supporting Information, Table S2). Microsatellite markers with levels of missing data in population 1, Mongolia, above 35% were excluded from all analyses.

A Bayesian clustering analysis was performed, on the reduced microsatellite panel (13 loci), using RMavericK v.1.0.5 (Verity & Nichols, 2016) to provide an estimate of the number of distinct genetic groups present amongst the *S. t. mongolica* and *S. t. tatarica* (Kazakhstan and Kalmykia regions) individuals. This approach assumes a Hardy–Weinberg (HW) equilibrium and linkage equilibria among loci, introduces population structure and assigns populations that are not in linkage equilibrium using a Markov chain Monte Carlo (MCMC) algorithm to estimate the number of populations (K). The algorithm was run three times, using both the admixture and the non-admixture model, for each K to ensure convergence of values, and with a burn-in of 200 000 repetitions, 2 000 000 repetitions after burn-in and K = 1–5, the non-admixture model was the best fit for the data (higher log likelihood). A different analysis following the same parameters as described above was done for a subsampled data set including 11 individuals for each subspecies, with same number of individuals per subspecies. Subsampling was done using an online random number generator.

RESULTS

We amplified and sequenced 880 base-pairs (bp) of the mtDNA CR in 62 *S. t. mongolica* (a total success rate of 81.6% for this subspecies) and 12 *S. t. tatarica* individuals (100% of the individuals tested). High success rates were achieved for the three kinds of tissue used: skin (95%), dried umbilical cord (76.9%) and hair samples (100%). The full data set contained 23 segregating (S) sites, defining 14 haplotypes (h, Genbank accession numbers OL979121–OL979134). No haplotypes were shared between the two subspecies, with three segregating sites separating them (Fig. 2A). The Mongolian population (N = 62) contained six segregating sites and a nucleotide diversity of 0.00655. Four haplotypes were found for the Mongolian populations and ten haplotypes for *S. t. tatarica*. The most frequent haplotype was H1 found in the Mongolian subspecies (N = 32), followed by H2 which was almost as frequent (N = 26).

A total of ten haplotypes were identified among the *S. t. tatarica* individuals. The Kazakhstan population contained 40 segregating (S) sites that defined five haplotypes (h) and the Kalmykia subpopulation contained 15 segregating (S) sites that defined five haplotypes (h). There are no shared haplotypes between the two populations Kazakhstan (N = 5) and Kalmykia (N = 7). Haplotype diversity (Hd), nucleotide diversity (π) and nucleotide differences (K) are higher for the Kazakhstan subpopulation (1.000, 0.03350 and 20.600) than the Kalmykia subpopulation (0.857, 0.01959 and 6.190), confirming previous results (Kholodova *et al.*, 2006; Campos *et al.*, 2010). Diversity statistics for the subspecies (Hd, π and K, respectively) are higher for *S. t. tatarica* (0.955, 0.02076 and 6.561) than *S. t. mongolica* (0.581, 0.00655 and 1.676).

As some of the sequences from the Mongolian subspecies were considerably shorter, we ran a separate analysis on a smaller data set (N = 57) that contained 15 segregating sites and defined eight haplotypes (Fig. 2B).

Nineteen microsatellites were screened for all samples, five of which resulted as monomorphic (Supporting Information, Table S2). The number of alleles per locus in population 1 (Mongolia) varied from one to 12 with six loci with three different alleles (Supporting Information, Table S2). We found no significant deviations from Hardy–Weinberg proportions after Bonferroni corrections in population 1 (Mongolia) and population 2 (Kalmykia); however, we found deviation from Hardy–Weinberg proportions after Bonferroni corrections in population 3 (Kazakhstan), which can be explained by the small number of individuals tested (Table 1; Supporting Information, Table S2). Expected and observed heterozygosity were low for both *S. t. mongolica* (He = 0.325, Ho = 0.290) and *S. t. tatarica* subspecies (He = 0.215, Ho = 0.313 for Kalmykia and He = 0.274, Ho = 0.365 for Kazakhstan). Fst values between the three populations are low, 0.029 between the Mongolian subspecies and the one from Kazakhstan, 0.042 between the Mongolian and the Kalmykian populations, and 0.109 between the two populations of *S. t. tatarica*. The inbreeding coefficient (F) for the Mongolian subspecies is 0.202. The two *S. t. tatarica* subpopulations present a negative F, -0.436 for Kalmykia and -0.308 for Kazakhstan. The Shannon information index values are 0.628 in the Mongolian saiga, 0.342 in Kalmykia and 0.463 in Kazakhstan (Table 1). Three microsatellites (Sta 02, Sta 03 and Sta 30), monomorphic for *S. t. mongolica*, were described as polymorphic for *S. t. tatarica* (Nowak *et al.*, 2014). Two microsatellites (Sta 05 and Sta 69) showed to be monomorphic for both subspecies (Supporting Information, Table S2).

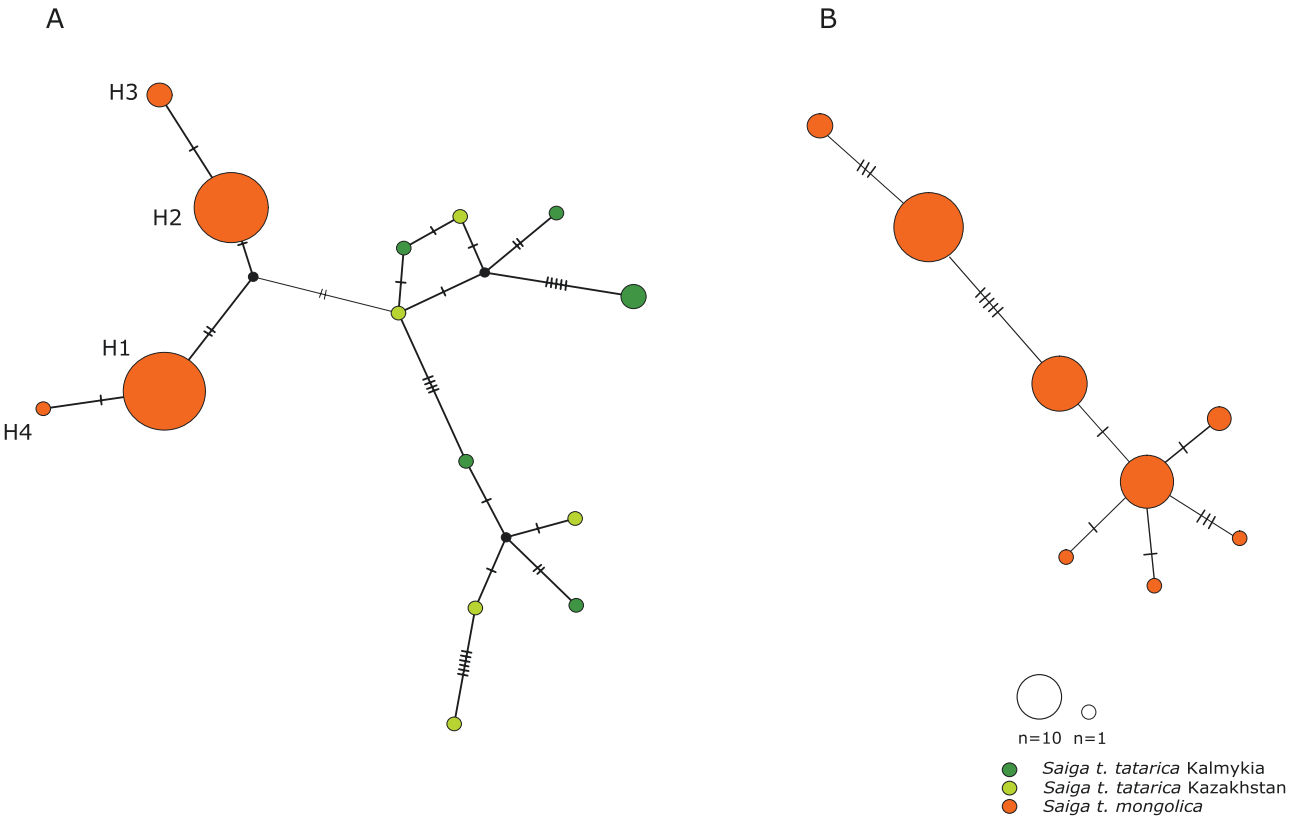

Figure 2. A, median joining network of 880 bp of the control region for 62 *S. t. mongolica* and 12 *S. t. tatarica* (drawn in Inkscape v.1.0.1). B, median joining network of 647 bp of the control region for 57 *S. t. mongolica*. Circle size represents number of sequences per haplotype. Dashes represent number of mutation steps between haplotypes.

Table 1. Diversity indices for saiga. Mean and standard error (SE) over loci for each population

Population		*N*	Na	Ne	I	Ho	He	uHe	F
Pop1	Mean	77	3.77	1.80	0.628	0.290	0.325	0.327	0.202
	SE		0.67	0.32	0.138	0.072	0.065	0.065	0.099
Pop2	Mean	6	1.69	1.46	0.342	0.313	0.215	0.235	-0.436
	SE		0.26	0.18	0.118	0.108	0.071	0.078	0.080
Pop3	Mean	5	2.08	1.60	0.463	0.365	0.274	0.317	-0.308
	SE		0.33	0.22	0.124	0.102	0.069	0.080	0.106
Total	Mean	88	2.51	1.62	0.478	0.323	0.271	0.293	-0.091
	SE		0.30	0.14	0.074	0.054	0.039	0.043	0.071

Pop1: Mongolia; Pop2: Kalmykia; Pop3: Kazakhstan; *N*: sample size; Na: no. alleles; Ne: no. effective alleles; I: Shannon's information index; Ho: observed heterozygosity; He: expected heterozygosity; uHe: unbiased expected heterozygosity; F: inbreeding coefficient.

Microsatellite screening of the six putative twin sets reveals allele differences in several pairs, suggesting saiga can give birth to dizygotic twins (Supporting Information, Table S1).

Cluster analysis performed with RMavericK on the microsatellite data for the two subspecies reveals the presence of a single population (K = 1, Fig. 3), i.e. no structure is found between the two subspecies at the nuclear level. The same result was obtained for a subsampled data set that includes the same number of individuals for each subspecies (Supporting Information, Fig. S1). However, these results should be taken with some caution given the small number of individuals tested for *S. t. tatarica*.

Figure 3. Inference of population structure of saiga estimated in RMavericK, for values of K from 1 (top) to 5 (bottom). Pop1: *S. t. mongolica*, Mongolia; Pop2: *S. t. tatarica*, Kalmykia; Pop3: *S. t. tatarica*, Kazakhstan.

DISCUSSION

Absence of population structure within the Mongolian saiga subpopulations

Our results demonstrate that the Mongolian saiga presents low mitochondrial diversity with only eight different mitochondrial haplotypes defined by 647 bp in a total of 57 individuals, separated by 15 segregating sites—similar levels of genetic diversity were obtained on previous studies based on smaller fragments of the mtDNA control region (Kholodova *et al.*, 2006; Sod-Erdene *et al.*, 2017). None of the haplotypes identified in Mongolian saiga were detected in the populations from Kazakhstan (Betpak-Dala) or Russia (Kalmykia), suggesting isolation of the two subspecies at the mitochondrial level. They were, however, only separated by two segregating sites, this would be expected as the subspecies are nowadays geographically isolated due to the presence of the Altai Mountain Range. We could not identify any genetic structure at the mitochondrial

level between the two sampled regions in Mongolia (Sharga Gobi and Khuis Gobi). This is consistent with the expectation that there is gene flow between these two regions; thus, gene flow with the third region, Dorgon Steppe, should also be expected. Recently, this has been confirmed using GPS tracking technology (Chimeddorj *et al.*, 2016). Similar results were obtained for the nuclear dataset using RMavericK, a Bayesian clustering analysis, confirming the presence of a single evolutionary unit within our Mongolian data set.

Genetic analyses reveal low genetic diversity in saiga

In comparison with other antelopes the saiga mtDNA nucleotide diversity is similar to that observed in modern Przewalski's gazelle (*Procapra przewalskii*) (Lei *et al.*, 2003; Yu *et al.*, 2017), which also experienced a decline in population size and, at present, is divided into four isolated subpopulations (Olson, 2010). Two other Mongolian gazelles that also live on a steppe ecosystem, the goitered gazelle (*Gazella subgutturosa*) and the Mongolian gazelle (*Procapra gutturosa*), have also experienced a population decline in recent times (Milner-Gulland & Lhagvasuren, 1998; Zachos *et al.*, 2010; Okada *et al.*, 2015; Khosravi *et al.*, 2018). The Mongolian gazelle suffered a 75% decline in habitat availability/range (Lhagvasuren & Milner-Gulland, 1997), but still has high mobility, moving long distances to search for resources and shelter. In comparison with saiga antelope, both gazelle species showed a two-fold higher expected heterozygosity. The Mongolian gazelle and the Mongolian saiga are nomadic species, but the Mongolian saiga, unlike *S. t. tatarica*, does not have large-scale migrations, as a large part of the species' range is enclosed by the massive Altai Mountains (Buuveibaatar *et al.*, 2013b).

Microsatellite analyses revealed similar genetic diversity results, with most loci having low heterozygosity (Ho/He) and a low number of alleles per locus (Na) (Table 1), which would be expected in a population that has suffered a large and rapid decline in very recent times. We have compared observed and expected heterozygosity levels for the Mongolian subspecies, with the ones published by Nowak *et al.* (2014) for *S. t. tatarica* subspecies, and with the exception of Sta44 and Sta80 the values were higher for *S. t. tatarica*. This may be a consequence of the smaller population sizes of the Mongolian subspecies, and the recurring population bottlenecks, as the population size of Mongolian saiga has, more than once, been lower than 1000 individuals (Chimeddorj *et al.*, 2008).

The low genetic diversity present in the Mongolian subspecies is a risk for the survival of the subspecies. Being a small, isolated population with low levels of genetic diversity, the Mongolian saiga is highly susceptible to both stochastic demographic and genetic risks, that can reduce fitness and compromise long-term persistence, as well as disease outbreaks. In 2017, after these samples were collected, 54% of saiga in Mongolia were wiped out by PPR (Pruvot *et al.* 2020). According to WWF Mongolia, this specific outbreak mostly affected males and calves, massively altering herd structure (Chimeddorj B., pers. comm.).

Outbreaks like this are a major extinction risk for the Mongolian populations given the way of life of nomadic people who live and travel with livestock (mostly goats and sheep). These may carry several viruses, like PPR, that can overlap with species ranges and spill over to the population. Mass mortality events (MME) have also affected populations in Kazakhstan, the latest in 2015 was caused by the bacteria *Pasteurella* sp. (Kock *et al.*, 2018; Fereidouni *et al.*, 2019).

Almost identical *Pasteurella* strains have been found in both healthy and sick populations of the species over decades which suggests that latent infections with endemic strains may occur (Kock *et al.*, 2018). However, the reason why this happens, i.e. what makes a strain suddenly virulent, is unknown. *In vitro* studies of bacterial virulence could reveal the mechanisms behind this process or the emergence of new virulent strains.

Absence of genetic differentiation between *S. t. tatarica* and *S. t. mongolica*

The lack of genetic structure between *S. t. tatarica* and *S. t. mongolica*, shown by the cluster analysis performed with RMavericK and by low Fst values, may be indicative of reduced time since separation of both subspecies. Lack of genetic differentiation between both subspecies can also be a result of reduced resolution due to the small number of individuals sampled from *S. t. tatarica*. In this way, an increased sampling effort, combined with genetic studies increasing the number of analysed nuclear markers, ideally using nuclear genomic information, would be necessary to elucidate the genetic structure of the subspecies and to evaluate whether both should be raised to species level.

Even though there are now several other reduced representation genome sequencing methods, like RADSeq (Restriction Site Associated DNA), ddRADSeq (double digest RADSeq) or GBS (Genotype by Sequencing), we opted to use microsatellite markers in this study as: (a) there is a set of markers recently developed for the species; (b) they are fast evolving markers, allowing for mutations to accumulate in short time spans, being ideal to study population viability; (c) they are widely distributed throughout the genome, especially in the euchromatin of eukaryotes, and coding and non-coding nuclear, and organellar DNA;

and (d) the nature of our samples, most of them from dried umbilical cord, also favour the use of PCR-based techniques due to the highly fragmented nature of the extracted DNA. This fact hindered the use of RAD-Seq and GBS that require high molecular weight genomic DNA to ensure the reproducibility of RAD or GBS tags in most samples (Andrews *et al.*, 2016).

The lack of structure is consistent with a population that has suffered a genetic bottleneck. In addition to population number fluctuations, poaching for horns can also decrease the genetic variation among Mongolian saiga. Previous studies have shown that population size can drastically decline after a catastrophic drop in the number of adult males (Rodríguez-Muñoz *et al.*, 2015), as it leads, among other things, to reproductive collapse with the decline in the number of pregnancies (Milner-Gulland *et al.*, 2003).

Inbreeding coefficient in the Mongolian saiga

One potential threat to the viability of the Mongolian subspecies is inbreeding, as inbreeding depression can lead to the collapse of a population. Our study shows that the Mongolian population has an excess of homozygotes (F = 0.202), presumably due to continuous population fluctuations and the small effective population size. The low genetic differentiation in the Mongolian saiga makes the population vulnerable to stochastic events like climate change or disease outbreaks. In fact, the PPR virus outbreak resulted in the death of at least 5500 saiga individuals, comprising more than half of the Mongolian saiga population (Pruvot *et al.*, 2020).

Dizygotic twins in saiga antelope

The saiga is a very prolific animal, and because both maturation and gestation time is short, females can give birth within the first year, and from the second reproduction cycle births of twins are common: between 25% and 65% of females have twins (Kühl *et al.*, 2007), a trend also found in other small ungulates (Robbins & Robbins, 1979). They are considered 'big spenders', as litter mass at birth is on average 16.9% of maternal body mass, putting the saigas among the species with highest reproductive outputs among ungulates (Robbins & Robbins, 1979). Some of the samples collected for this study are from putative twin births (see Supporting Information, Table S1). In some species, like the goitered gazelle, which has a similar hiding reproductive strategy as the saiga antelope, adult females in their reproductive prime (between the ages of 3 and 7 years) typically bear twins, while young and old females produce a single offspring. Twins can represent up to 75% of pregnancies in adult females when environmental conditions are favourable; however, this number decreases when conditions are unfavourable (Kingswood & Blank, 1996). This strategy is probably also used by saiga to quickly increase population numbers when conditions are favourable in an environment of great instability and abrupt change as in the arid plains of Central Asia. Monozygotic twins occur when a single fertilized egg splits and develops into two individuals, and therefore their DNA is identical; as opposed to dizygotic twins that originate from two different fertilized eggs and have the same genetic relationship as ordinary siblings. Our results suggest that when saiga antelope gives birth to twins, they are usually dizygotic twins. Five out of six pairs of twins were dizygotic, four with at least one allele difference, and a set of male/female calves. Given that the birth of twins seems to be a strategy from these small antelopes to rapidly increase population size in good environmental conditions the presence of dizygotic twins was to be expected. More samples from twins are however needed to confirm the trend observed in our results.

Saiga tatarica mongolica conservation

Mass mortality events (MMEs) are not a new threat to the species, saiga populations have been affected by them since time immemorial. During harsh winters, saiga mortality can be up to 50%, followed by recovery in the next 2-3 years (Bekenov *et al.*, 1998). Due to these mass mortality events, caused by harsh winters, *Pasteurella* sp. or PPR virus and, in more recent times, increased poaching, saiga effective population sizes have always fluctuated immensely.

Hence, saiga management should be done with the premise that there is a high probability of occurrence of MMEs in the near future. Preventive measures that include the vaccination of livestock against PPR, responsible for the demise of the Mongolian population in 2018, should be put into practice specially in Mongolia where livestock husbandry is by far the most important local industry. States across the natural range of this species should also continue to apply strong antipoaching actions so populations are large enough to be able to surpass the large-scale mortality. A sustainable landscape approach should also be considered, avoiding the construction of roads, irrigation channels or other infrastructures within the species range allowing for the peaceful coexistence of people, livestock and the endangered saiga antelope. Our results show a lack of structure within *S. t. mongolica*, confirming the results obtained by Berger *et al.* (2008) that there is movement between subpopulations. This indicates that measures put in place by conservationists in Mongolia, where movement routes are protected, seem to be working.

Here, we showed that the Mongolian saiga subspecies is characterized by low genetic diversity at the mitochondrial level, with no exclusive haplotypes in either of the Mongolian subpopulations included in this study, suggesting some level of gene flow between them. At the nuclear level, *S. t. mongolica* also shows low levels of genetic diversity with the number of alleles per locus between one and eleven, and no genetic cluster structure detected. In this way, despite the low genetic diversity present in the Mongolian subspecies, the gene flow between the different regions in the Gobi Desert seems to be beneficial for the long-term survival of the species, implying that connectivity corridors between the different regions are essential and should be protected.

With the current sampling it is difficult to recommend any conservation strategy for *S. t. tatarica*, as the number of samples used in this study is small. An increased sampling effort would be recommended, in order to better assess the subpopulation structure in both subspecies. Furthermore, it would be ideal to increase the number of analysed markers in order to increase genetic resolution, which would help to generate better estimates of genetic diversity and inbreeding coefficients as well as to further elucidate the relationship between saiga subspecies.

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DATA AVAILABILITY

The microsatellite genotypes are available in the Supporting Information.

REFERENCES

Andrews KR, **Good JM**, **MIller RM**, **Luikart G**, **Hohenlohe PA. 2016.** Harnessing the power of RADseq for ecological and evolutionary genomics. *Nature Reviews Genetics* **17:** 81–92.

Bandelt HJ, **Forster P**, **Röhl A. 1999.** Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16:** 37–48.

Bekenov AB, **Grachev IA**, **Milner-Gulland EJ. 1998.** The ecology and management of the saiga antelope in Kazakhstan. *Mammal Review* **28:** 1–52.

Berger J, **Buuveibaatar B**, **Mishra C. 2013.** No globalization of the cashmere market and the decline of large mammals in Central Asia. *Conservation Biology* **27:** 679–689.

Berger J, **Young JK**, **Berger KM. 2008.** Protecting migration corridors: challenges and optimism for Mongolian saiga. *PLoS Biology* **6:** e165.

Buuveibaatar B, **Fuller TK**, **Fine AE**, **Chimeddorj B**, **Young JK**, **Berger J. 2013b.** Changes in grouping patterns of saiga antelope in relation to intrinsic and environmental factors in Mongolia. *Journal of Zoology* **291:** 51–58.

Buuveibaatar B, **Fuller TK**, **Young JK**, **Berger J. 2014.** Calving location selection patterns of saiga antelope in Mongolia. *Journal of Zoology* **294:** 241–247.

Buuveibaatar B, **Young JK**, **Berger J**, **Fine AE**, **Lkhagvasuren B**, **Zahler P**, **Fuller TK. 2013a.** Factors affecting survival and cause-specific mortality of saiga calves in Mongolia. *Journal of Mammalogy* **94:** 127–136.

Campos PF, **Gilbert TMP. 2012**. DNA extraction from keratin and chitin. Shapiro B., Hofreiter M, eds. *Ancient DNA. Methods in Molecular Biology*, vol 840. 43–49. Humana Press.

Campos PF, **Kristensen T**, **Orlando L**, **Sher A**, **Kholodova MV**, **Götherström A**, **Hofreiter M**, **Drucker DG**, **Kosintsev P**, **Tikhonov A**, **Baryshnikov GF**, **Willerslev E**, **Gilbert MTP. 2010.** Ancient DNA sequences point to a large loss of mitochondrial genetic diversity in the saiga antelope (*Saiga tatarica*) since the Pleistocene. *Molecular Ecology* **19:** 4863–4875.

Carroll RL. 1988. *Vertebrate paleontology and evolution*. New York: WH Freeman.

Chimeddorj B, **Amgalan L**, **Buuveibaatar B. 2008.** Current status and distribution of the saiga in Mongolia. *Saiga News* **8:** 1–2.

Chimeddorj B, **Gantulga B**, **Fine AE**, **Enkhtuvshin S**, **Buuveibaatar B. 2019.** An update on the estimated population size of the Mongolian saiga. *Saiga News* **25:** 16–18.

Chimeddorj B, **Sergelen L**, **Buuveibaatar B. 2016.** Current status and distribution of the saiga in Mongolia. *Saiga News* **21:** 35–37.

Clark EL, **Munkhbat J**, **Dulamtsėrėn S**, **Baillie J**, **Batsaikhan N**, **Samiya R**, **Stubbe M. 2006.** *Mongolian red list of mammals*. London: Zoological Society of London.

Cui S, **Milner-Gulland EJ**, **Singh NJ**, **Chu H**, **Li C**, **Chen J**, **Jiang Z. 2017.** Historical range, extirpation and prospects for reintroduction of saigas in China. *Scientific Reports* **7:** 1–11.

Dulamtseren S, **Amgalan L. 1995.** Distribution and contemporary population of Mongolian saiga (*Saiga tatarica mongolica* Bannikov). In: *Natural conditions and biological resources of the Great Gobi Reserve. Ulaanbaatar: Ministry of Nature and Environment*, 45–51. The First Scientific Conference, Ulaanbaatar [in Mongolian].

Fereidouni S, **Freimanis GL**, **Orynbayev M**, **Ribeca P**, **Flannery J**, **King DP**, **Zuther S**, **Beer M**, **Höper D**, **Kydyrmanov A**, **Karamendin K**, **Kock R. 2019.** Mass die-off of saiga antelopes, Kazakhstan, 2015. *Emerging Infectious Diseases* **25:** 1169–1176.

IUCN. 2018. *Saiga tatarica. The IUCN Red List of Threatened Species. IUCN SSC Antelope Specialist Group 2018 e.T19832A50194357*. Available at: http://dx.doi.org/10.2305/IUCN.UK.2018-2.RLTS.T19832A50194357.en. Accessed 10 October 2019.

Jürgensen J, **Drucker DG**, **Stuart AJ**, **Schneider M**, **Buuveibaatar B**, **Bocherens H. 2017.** Diet and habitat of the saiga antelope during the Late Quaternary using stable carbon and nitrogen isotope ratios. *Quaternary Science Reviews* **160:** 150–161.

Kholodova M, **Milner-Gulland EJ**, **Easton AJ**, **Amgalan L**, **Arylov IA**, **Bekenov A**, **Grachev IA**, **Lushchekina AA**, **Ryder O. 2006.** Mitochondrial DNA variation and population structure of the Critically Endangered saiga antelope *Saiga tatarica*. *Oryx* **40:** 103–107.

Khosravi R, **Hemami MR**, **Malekian M**, **Silva TL**, **Rezaei HR**, **Brito JC. 2018.** Effect of landscape features on genetic structure of the goitered gazelle (*Gazella subgutturosa*) in Central Iran. *Conservation Genetics* **19:** 323–336.

Kingswood S, **Blank DA. 1996.** *Gazella subgutturosa*. *Mammalian Species Archive* **518:** 1–10.

Kock RA, **Orynbayev M**, **Robinson S**, **Zuther S**, **Singh NJ**, **Beauvais W**, **Morgan ER**, **Kerimbayev A**, **Khomenko S**, **Martineau HM**, **Rystaeva R**, **Omarova Z**, **Wolfs S**, **Hawotte F**, **Radoux J**, **Milner-Gulland EJ. 2018.** Saigas on the brink: multidisciplinary analysis of the factors influencing mass mortality events. *Science Advances* **4:** eaao2314.

Kohn MH, **Murphy WJ**, **Ostrander EA**, **Wayne RK. 2006.** Genomics and conservation genetics. *Trends in Ecology and Evolution* **21:** 629–637.

Kühl A, **Mysterud A**, **Erdnenov GI**, **Lushchekina AA**, **Grachev IA**, **Bekenov AB**, **Milner-Gulland EJ. 2007.** The 'big spenders' of the steppe: sex-specific maternal allocation and twinning in the saiga antelope. *Proceedings of the Royal Society B: Biological Sciences* **274:** 1293–1299.

Lei R, **Hu Z**, **Jiang Z**, **Yang W. 2003.** Phylogeography and genetic diversity of the Critically Endangered Przewalski's gazelle. *Animal Conservation* **6:** 361–367.

Leigh JW, **Bryant D. 2015.** popart: full-feature software for haplotype network construction. *Methods in Ecology and Evolution* **6:** 1110–1116.

Lhagvasuren B, **Milner-Gulland EJ. 1997.** The status and management of the Mongolian gazelle *Procapra gutturosa* population. *Oryx* **31:** 127–134.

Librado P, **Rozas J. 2009.** DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25:** 1451–1452.

Lushchekina AA, **Dulamtseren S**, **Amgalan L**, **Neronov VM. 1999.** The status and prospects for conservation of the Mongolian saiga *Saiga tatarica mongolica*. *Oryx* **33:** 21–30.

Milner-Gulland EJ, **Bukreeva OM**, **Coulson T**, **Lushchekina AA**, **Kholodova MV**, **Bekenov AB**, **Grachev IA. 2003.** Reproductive collapse in saiga antelope harems. *Nature* **422:** 135.

Milner-Gulland EJ, **Kholodova MV**, **Bekenov A**, **Bukreeva OM**, **Grachev IA**, **Amgalan L**, **Lushchekina AA. 2001.** Dramatic declines in saiga antelope populations. *Oryx* **35**.

Milner-Gulland EJ, **Lhagvasuren B. 1998.** Population dynamics of the Mongolian gazelle *Procapra gutturosa*: an historical analysis. *Journal of Applied Ecology* **35:** 240–251.

Nowak C, **Zuther S**, **Leontyev S**, **Geismar J. 2014.** Rapid development of microsatellite markers for the Critically Endangered saiga (*Saiga tatarica*) using Illumina® Miseq next generation sequencing technology. *Conservation Genetics Resources* **6:** 159–162.

Okada A, **Ito TY**, **Buuveibaatar B**, **Lhagvasuren B**, **Tsunekawa A. 2015.** Genetic structure in Mongolian gazelles based on mitochondrial and microsatellite markers. *Mammalian Biology-Zeitschrift für Säugetierkunde* **80:** 303–311.

Olson KA. 2010. A literature review of Mongolian gazelle (*Procapra gutturosa*) ecology from 1998 to present. *Mongolian Journal of Biological Sciences* **8:** 15–24.

Peakall P, **Smouse R. 2012.** GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics* **28:** 2537–2539.

Pruvot M, **Fine AE**, **Hollinger C**, **Strindberg S**, **Damdinjav B**, **Buuveibaatar B**, **Chimeddorj B**, **Bayandonoi G**, **Khishgee B**, **Sandag B**, **Narmandakh J**, **Jargalsaikhan T**, **Bataa B**, **McAloose D**, **Shatar M**, **Basan G**, **Mahapatra M**, **Selvaraj M**, **Parida S**, **Njeumi F**, **Kock R**, **Shiilegdamba E. 2020.** Outbreak of peste des petits ruminants virus among Critically Endangered Mongolian saiga and other wild ungulates, Mongolia, 2016-2017. *Emerging Infectious Diseases* **26:** 51–62.

Radwan J, **Kuduk K**, **Levy E**, **LeBas N**, **Babik W. 2014.** Parasite load and MHC diversity in undisturbed and agriculturally modified habitats of the ornate dragon lizard. *Molecular Ecology* **23:** 5966–5978.

Robbins CT, **Robbins BL. 1979.** Fetal and neonatal growth patterns and maternal reproductive effort in ungulates and subungulates. *The American Naturalist* **114:** 101–116.

Rodríguez-Muñoz R, **Del Valle CR**, **Bañuelos MJ**, **Mirol P. 2015.** Revealing the consequences of male-biased trophy hunting on the maintenance of genetic variation. *Conservation Genetics* **16:** 1375–1394.

Saiga Conservation Alliance. **2013.** Huge consignment of saiga horns seized in China. *Saiga Conservation Alliance*.

Sod-Erdene B, **Bolor-Oyut B**, **Battsetseg B**, **Tumennasan K**, **Ochirkhuyag B**, **Khulan J. 2017.** Comparative analysis of mitochondrial DNA of the Mongolian and Kazakhstan saiga. *Proceedings of the Institute of General and Experimental Biology* **33:** 246–252. [in Mongolian].

Spurgin LG, **Richardson DS. 2010.** How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. *Proceedings of the Royal Society B: Biological Sciences* **277:** 979–988.

Sugimoto T, **Ito TY**, **Taniguchi T**, **Lkhagvasuren B**, **Oyunsuren T**, **Sakamoto Y**, **Yamanaka N. 2018.** Diet of sympatric wild and domestic ungulates in southern Mongolia by DNA barcoding analysis. *Journal of Mammalogy* **99:** 450–458.

Verity R, **Nichols RA. 2016.** Estimating the number of subpopulations (k) in structured populations. *Genetics* **203:** 1827–1839.

Wilson DE, **Reeder DM. 2005.** *Mammal species of the world: a taxonomic and geographic reference*. JHU Press.

Yoshihara Y, **Ito TY**, **Lhagvasuren B**, **Takatsuki S. 2008.** A comparison of food resources used by Mongolian gazelles and sympatric livestock in three areas in Mongolia. *Journal of Arid Environments* **72:** 48–55.

Yu H, **Song S**, **Liu J**, **Li S**, **Zhang L**, **Wang D**, **Luo SJ. 2017.** Effects of the Qinghai-Tibet railway on the landscape genetics of the Endangered Przewalski's gazelle (*Procapra przewalskii*). *Scientific Reports* **7:** 1–13.

Zachos FE, **Karami M**, **Ibenouazi Z**, **Hartl GB**, **Eckert I**, **Kirschning J. 2010.** First genetic analysis of a free-living population of the threatened goitered gazelle (*Gazella subgutturosa*). *Mammalian Biology-Zeitschrift für Säugetierkunde* **75:** 277–282.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Sample details.
Table S2. Microsatellite genotyping.
Figure S1. Inference of population structure of saiga estimated in RMavericK, for a subsampled data set including 11 individuals for each subspecies.