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From social patterns to genetic structure: the reintroduced Asiatic wild ass (*Equus hemionus*) in the Negev Desert

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Abstract Genetic diversity is critical for the long-term viability of wildlife populations. While classical population genetic models assume random mating and dispersal, social behavior might violate these assumptions and shape genetic structure. This effect is especially relevant in small populations, where genetic drift and non-random mating can have pronounced impacts. We studied the reintroduced population of Asiatic wild asses (*Equus hemionus*) in the Negev Desert, characterized by fission-fusion social-structure, to investigate how social behavior influences genetic structure. We combined behavioral observations and non-invasive genetic sampling. DNA was extracted from fecal samples collected across the range of distribution around water sources (population's activity centers). Direct observations showed that only 24–37% of males were dominant (territorial males that breed), with some maintaining dominancy for at least four years, indicating a strongly polygynous mating system. Genetic analyses of mitochondrial and nuclear markers using F-statistics and a network-based approach (*Netstruct analysis*) indicated a fine-scale population genetic structure. This structure had developed over a small geographical range, suggesting either historical founder effects or contemporary limitations to gene flow. The long dominance tenures of territorial males and their high fidelity to water sources may reduce dispersal between activity centers leading to genetic differentiation. These findings highlight the role of social behavior, particularly mating system, in shaping population genetic structure. They emphasize the importance of long-term genetic monitoring and integrating social-structure into conservation planning. For example, management actions to enhance connectivity within the population could include adding water sources between activity centers to facilitate gene flow.

Keywords *Equus hemionus*; Asiatic wild ass; genetic structure; mating system; social structure; conservation

Introduction

Genetic diversity represents the adaptive potential of a population and therefore may influence the population's long-term viability and sustainability (Soule 1987; Frankham *et al.* 2002; Hughes *et al.* 2008). In many population-genetic models, random mating and between-population migration are assumed (Chesser 1991). However, in social species, this assumption rarely holds because social behavior usually generates non-random mating patterns and can strongly impact movement, dispersal, and recruitment. Importantly, through its direct effect on the mating system, sociality can affect the proportion of breeding individuals and their relative contribution to the gene-pool (reviewed in (Sugg *et al.* 1996; Storz 1999). In polygamous mating systems, where not all individuals contribute their genes to the next generation, genetic drift is typically increased, leading to a reduction of genetic diversity (e.g., in red howler monkey *Alouatta seniculus* (Pope 1992), the black-tailed prairie dog *Cynomys ludovicianus* (Sugg *et al.* 1996), and the semi-social shrew *Crocidura russula* (Balloux *et al.* 1998)). Theoretical considerations of genetic drift, taking into account both the inbreeding effective size (N_{ef}) and the variance effective size (N_{ev}), which reflect the vulnerability of a population to inbreeding and genetic drift

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(Wright 1931), suggest that mating systems of polygyny or polyandry can substantially reduce the ratio of effective size to census population size compared to promiscuous mating or monogamy (Parker & Waite 1997). Additionally, through the effect of social behavior on movement and space use patterns, sociality can have a major effect on gene flow, founder events, and genetic drift, and hence on the levels of genetic diversity and population structure (Hewitt & Butlin 1997; Storz 1999).

The effect of sociality in mammals on their population genetics is relatively well studied in social species in which females and males form permanent breeding groups, which is the more common form of sociality in mammals. These include monogamous species (e.g., the greater white-toothed shrew, (Balloux *et al.* 1998; Favre *et al.* 1997); the fat-tailed dwarf lemur, (Fredsted *et al.* 2007) and the mound-building mouse, (Poteaux *et al.* 2008), and polygamous species that live in stable breeding groups (e.g., macaques, baboons and vervet monkeys, (Melnick 1987); the red howler monkey, (Pope 1992); the European wild rabbit, (Surridge *et al.* 1999); the black-tailed prairie dog, (Dobson *et al.* 2004) and the wild boar, (Poteaux *et al.* 2009). In contrast, the extent to which sociality can impact genetic diversity and population structure in social species with no permanent breeding groups has been examined in several studies (Coltman *et al.* 2003; Archie *et al.* 2008), but further research is needed. Understanding this effect is particularly important in small and threatened populations, as these populations are prone to extensive loss of genetic diversity. However, collecting behavioral and genetic data for such populations is challenging because small populations are usually rare, elusive, and sensitive.

The Asiatic wild ass (*Equus hemionus*) is an elusive near-threatened species (Kaczensky *et al.* 2015) that displays resource-defense polygyny and fission-fusion social structure (Saltz *et al.* 2000; Renan *et al.* 2018). In this social structure, females and their young tend to live in non-stable groups, and dominant males defend high-quality territories where female groups come to feed and mate. The territorial dominant males are solitary, while the subordinate non-territorial males live in unstable all-male bachelor groups (Klingel 1975; Rubenstein 1986; Moehlman 1998). This social system is characterized by unstable social groups, as females frequently change their group composition, sometimes within the same day (Renan *et al.* 2018), and dominant males tend to mate with multiple females during each breeding season (Klingel 1998; Moehlman 2002).

The Asiatic wild ass population of the Negev Desert, Israel, was reintroduced between the years 1982 and 1993. Following reintroduction, the population naturally expanded its geographical range, and the current population is estimated to be above 300 individuals (Zecherle *et al.* 2020). The population is distributed in large parts of the arid regions of Israel, where permanent water sources, which can serve for drinking throughout the year, are limited. During the hot summer months, which coincide with the breeding season, most of the population tends to stay near these few water sources. Three artificial water sources were established by the Israel Nature and Parks Authority (INPA) during 1980–90s to support the population: in Makhtesh Ramon, in Wadi Paran and in the Negev Highlands (Fig. 1). High feces densities were documented around these artificial water sources, as well as in Wadi Ashosh in the Arava Valley, and these four sites were considered as the main population activity centers (Nezer *et al.* 2017; Fig. 1). The relatively small population size, the complex social behavior, and the partitioning of the population to subpopulations around the water sources, provide an opportunity to study the effect of sociality on the population genetics of small populations with no stable breeding groups.

In this study, we combine five years of behavioral observation with non-invasive genetic sampling to examine the effect of the Asiatic wild ass social behavior on the genetic structure of the reintroduced population. We aim to explore the observed level of polygyny and to examine the dominance stability of males, comparing it to the detected genetic mating system (Renan *et al.* 2015) in order to test whether the dominant-territorial males are indeed the main contributors to the gene pool in the population. We further evaluate the spatial genetic structure of the Negev population to examine the potential effect of the social behavior, in this complex social system, on population genetic features.

Materials and Methods

The study population

The Asiatic wild ass was once abundant in western Asia, including the Negev desert of Israel, but declined throughout its range due to hunting and habitat loss. The local subspecies, *E. h. hemippus*, endemic to the Middle East, became extinct in the early 20th century (Harper 1945). In 1968 a breeding core was established at the Hai-Bar Yotvata Reserve from 11 individuals from the Iranian (*E. h. onager*, 3 M, 3 F) and the Turkmenian (*E. h. kulan*, 2 M, 3 F) subspecies (Zecherle *et al.* 2021). Between 1982 and 1993, the *E. hemionus* spp. was reintroduced into the Negev desert by the INPA in Ein Saharonim, Makhtesh Ramon (n=28), and in Wadi Paran (n=10), about 35 km south of Ein Saharonim (Saltz & Rubenstein 1995; Sinai 1994) (Fig. 1). During the late 1990s, the population naturally expanded its geographical range north of Makhtesh Ramon, to the Negev Highlands and east, to the Arava valley. Today, most of the population occurs in the Negev Highlands. The Negev Highlands are a part of the Irano-Turanian phytogeographical region, which is characterized by cold winters and hot, dry summers, and dwarf-shrub steppe vegetation (Berman 1989). The rest of the population occurs in the southern Negev and the Arava, which lie within the Saharo-Arabian and Sudano-Zambesian regions. Both these regions are characterized by low annual rainfall rates and mainly runnels vegetation (Danin *et al.* 1975).

Our null hypothesis was that there is no genetic structure in the Asiatic wild ass population within its relatively small distribution range (around 8,000 km^2). This is due to the fission-fusion social structure (Renan *et al.* 2018), which may facilitate gene flow among subpopulations; the relatively short time that have passed since

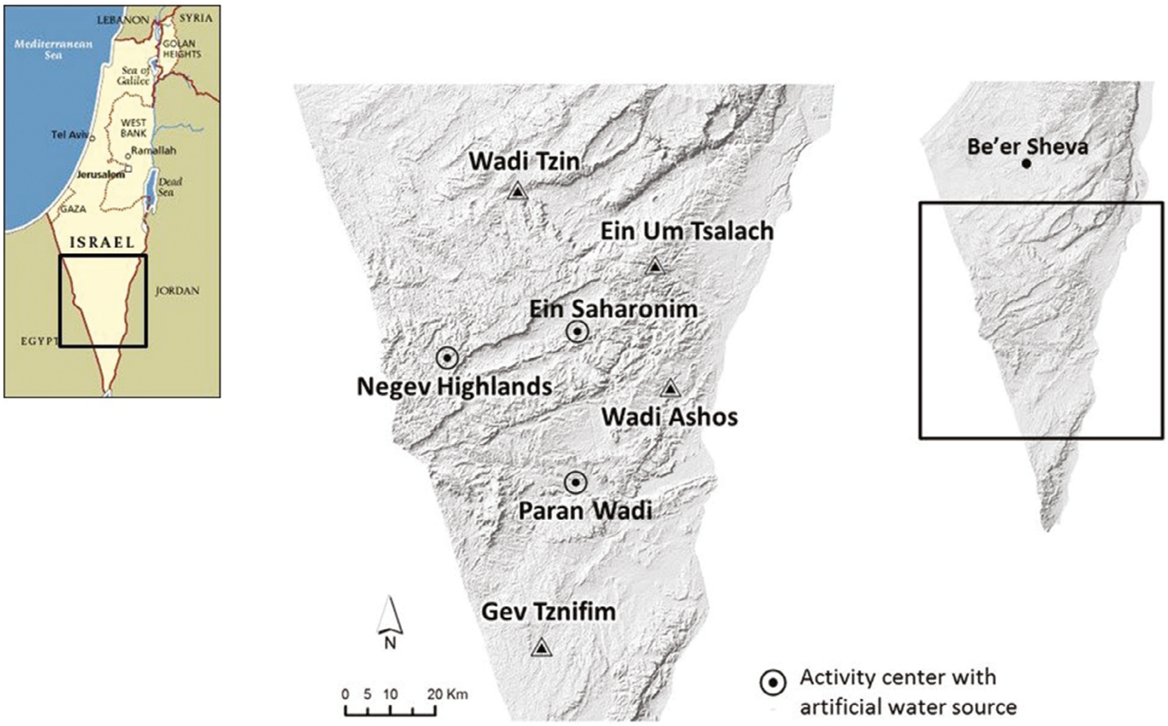

Figure 1. The sampling sites within the Asiatic wild ass distribution range in the Negev desert. In three of the sampling sites, there is an artificial water source, while in the vicinity of the others, there is a natural spring or an ephemeral desert pool.

the reintroduction (only four generations ago, Saltz & Rubenstein 1995); and because the wild ass is a large mammal that can walk more than 20 km per day (Saltz *et al.* 2000. However, the resource-defense polygyny of the wild ass population in this arid zone may lead to the formation of fine scale genetic population structure. Specifically, during summer, which is also the mating season, lactating females must drink at least once a day (Saltz *et al.* 2000; Giotto *et al.* 2016) and hence, are expected to show high fidelity to the water sources. If females return yearly to the same water source, a fine-scale population structure among females could form. Moreover, in a previous study on the genetic mating system of the Negev population, we found a genetically strong polygynous mating system in which less than 25% of all males participate in the mating process in each generation (Renan *et al.* 2015). In addition, Saltz *et al.* (2000) found that the dominant-territorial males set up their territories near water sources that attract females, and by doing so, they increase their mating opportunities. If the dominant-territorial males obtain most of the mating opportunities and are the main contributors to the gene pool (i.e., the genetic contribution of non-territorial male is minimal), and if their dominance tenure is relatively long (i.e., territories are being held for several years), their strong site fidelity may result in local breeding and limited gene flow among activity centers, accelerating the formation of population structure in the Negev population.

Behavioral data collection and analysis

The behavioral observations were conducted during four consecutive breeding seasons (June to October, 2010–2013 details in Renan *et al.* (2018)). In brief, direct observations were conducted near the permanent water source at the most populated activity center of the Negev Highlands (Fig. 1). Wild asses were filmed with a video camera (Sanyo Xacti VPC-FH1 HD, x30). Group compositions, including number of individuals, individuals' age categories (adult, sub-adult or juvenile), and individual's sex (when possible), were documented. A unique individual identification profile was generated for all individuals with distinguishable natural marking (similar to Van Dierendonck *et al.* 1996) by analyzing the close-ups of video films. Unique individual profiles were searched in all video films for multiple observations (similar to Feh *et al.* 2001).

To examine the level of polygyny, we calculated, for each year, the fraction of observations of solitary-dominant males out of all males observed. For groups with a majority of males, we assumed that non-identified adults in the group are male based on previous observations by Renan *et al.* (2018). Observations in which the sexes of most (50%>) individuals in the group were not determined, the groups were excluded from further analyses.

We examined the dynamics of male groups by monitoring identified males throughout time and documenting their group sizes. In order to examine the stability of the males' dominant position, we selected relatively easy to recognized male individuals that had highly distinctive marks (e.g., cut tail, bite scar, broken ear etc.) and that were dominant during 2010, 2011 or 2012, and followed their social status for another two years of study (this included additional data collected in 2014).

Genetic sampling, mitochondrial DNA sequencing and microsatellite genotyping

The genetic study included 242 samples: 219 fecal samples, 11 blood samples, and 12 tissue samples. The fecal samples were collected at the four main activity centers of the Negev wild ass population: Makhtesh Ramon, Wadi Paran, Wadi Ashosh and the Negev Highlands (Fig. 1), between Sept. 2012–Oct. 2013. Only fresh feces were collected and preserved according to the protocol established by Renan *et al.* (2012): the outer layer of the feces was wiped off using cotton swabs and preserved in 50 ml polypropylene tubes. The samples were transferred to the freezer (-20 °C) within a few hours after collection and extracted the following day. The blood and tissue samples were obtained from five collared individuals and from 18 road-killed individuals. These samples were stored at -20 °C until extraction. Fecal samples were extracted using a QIAamp® DNA Stool Mini Kit (QIAGEN, Cat. No. 51504) with several modifications according to (Renan *et al.* 2012). DNA from blood or tissue samples was extracted using QIAamp DNA Mini Kit (QIAGEN, Cat. No. 51304) following the manufacturer's instructions.

For the mitochondrial DNA (mtDNA) analysis we amplified 385 bp of the 12S1 locus following Gueta *et al.* (2014). PCR amplification protocols are detailed in Appendix 1. According to the procedure developed by Gueta *et al.* (2014), we initially used a restriction enzyme, NcoI (New England Biolabs, UK), and PCR products that were digested by the enzyme were determined as haplotype 1. In order to distinguish between the other haplotypes, PCR products that were not digested by the enzyme were sequenced by ABI 3730XL DNA analyzer (Applied Biosystems) and aligned using GENEIOUS software (Drummond *et al.* 2009). As a control, 10% of the samples were sequenced twice.

All samples were amplified using eight microsatellite loci (HMS2, HMS3, HMS6, AHT4, HTG4, LEX74, COR070, and UM11), which were shown to have high amplification success, low genotyping error and were polymorphic. PCR amplification protocols are in Renan *et al.* (2015). Genotypes of blood and tissue samples were manually assigned based on two consensual scores for each locus. To genotype individuals using the non-invasive fecal extracts, we used the multi-stage, multi-tubes approach (Navidi *et al.* 1992; Taberlet *et al.* 1996) with few modifications. First, three positive amplifications for each extract were carried out and scored. The individual was assigned as heterozygous if each of two alleles was scored at least twice, according to Taberlet *et al*. (Taberlet *et al.* 1996) or homozygous if three consensual homozygous scores were found. In cases where a final genotype could not be concluded based on these three amplifications, two additional amplifications were performed. A heterozygous individual was assigned when each of two alleles was scored at least three times out of five replicates, while a homozygous individual was assigned if four out of five consensual homozygous scores were found. Extracts that we could not genotype according to five amplifications were discarded from the analysis of that specific locus. To test the reliability of scoring homozygous individuals based on three amplifications, we selected a subset of 15% of the extracts (per locus) that were assigned as homozygous and performed two additional amplifications. When comparing the scores of the homozygous samples based on the five amplifications vs. the three amplifications, all homozygous genotypes remained homozygous.

Variability of microsatellite markers

Samples genotyped for at least seven out of the eight microsatellites were included in the analysis (n=97). Data for each locus were tested for linkage disequilibrium and for deviations from Hardy–Weinberg equilibrium (HWE) using the software ARLEQUIN version 3.5.1.3 (Excoffier & Lischer 2010). The loci were also tested for scoring errors due to stuttering, allelic dropout, and for the presence of null alleles, performed with MICRO-CHECKER version 2.2.3 (van Oosterhout *et al.* 2004). For each locus, the software GenAlEx 6.41 (Peakall & Smouse 2006) was used to estimate the number of alleles (*NA*), effective allele number, observed and unbiased expected heterozygosity under HWE (H_O and H_E, respectively), and the inbreeding coefficient as a deviation from HWE expectations. The probability of identity P_{ID} and the more conservative $P_{ID\text{-}sibs}$ (Waits *et al.* 2001) were calculated using GenAlEx 6.41 (Peakall & Smouse 2006) in order to check whether the genotypes could be used for discriminating among individuals.

Genetic structure

We studied the genetic structure of the Negev population using three approaches: 1) F-statistics, which rely on pre-analysis grouping of individuals to subpopulations; 2) a model-based clustering approach, which assigns individuals to subpopulations without pre-analysis grouping; and 3) a model-free network-based method for population assignment, which does not assume a pre-defined biological model.

F-statistics analysis. Because mtDNA is maternally inherited and microsatellites are autosomal markers that are inherited from both parents, we analyzed the genetic structure of both the mtDNA and the microsatellite datasets to enable the comparison between female-mediated genetic structure and the genetic structure of the entire population. For these analyses, we pre-defined the four activity centers as subpopulations. We performed an AMOVA (Φ_{PT}) on the mtDNA data (n=200) to test whether there is a significant difference in the haplotype frequencies among sites, and pairwise Φ_{PT} values were computed to examine the genetic differentiation patterns of the sites. The analysis was conducted with the software GenAlEx 6.41 (Peakall & Smouse 2006), set for haploid data, and permutation tests were used to determine the significance of Φ_{PT} values. Using the microsatellite data (n=97), an AMOVA (F_{ST}) test was performed to examine whether there is a significant difference in allele frequencies among the four sites. Pairwise F_{ST} values were calculated to test significant differences between two specific sites using the software ARLEQUIN version 3.5.1.3 (Excoffier & Lischer 2010). In addition, AMOVA (Φ_{PT}) and pairwise Φ_{PT} were calculated using *Codom-genotypic* distance option implemented in GenAlEx 6.41 (Peakall & Smouse 2006) to enable a comparison between the outputs of the mtDNA data and the microsatellites data analysis. For this purpose, we also used a subset of the mtDNA dataset (n=79), including only samples identified as unique individuals based on microsatellite genotypes. This conservative approach minimizes potential biases arising from the recapture of the same individuals when using non-invasive sampling. The significance of the AMOVA F_{ST} and Φ_{PT} values were calculated using permutation tests.

Model-based clustering. For identifying population structure without pre-analysis grouping of individuals, we used the model-based clustering method implemented in STRUCTURE 2.3.4 (Pritchard *et al.* 2000). We used the LOC-PRIOR model that gives higher prior weights to clusters that correlate with the sample location (Hubisz *et al.* 2009), because it was designed to reveal weak population structuring and, hence, it is more suitable for our system. The number of possible genetic clusters (K) was set from 1 to 7, and 20 independent runs for each *K* were performed using the admixture model and the correlated allele frequencies model, with burn-in period length of 100,000 iterations and number of MCMC repeats (Pritchard *et al.* 2000). We used the online program Structure Harvester (Earl 2012) to determine which K values is most supported by the analysis (Evanno *et al.* 2005). Replicate runs for each K were aligned and summarized using the program CLUMPAK version 1.1 (Kopelman *et al.* 2015).

Model-free network-based clustering. To test for genetic signals of differentiation, we also used a network-based approach designed to identify clusters in networks constructed from between-individual genetic similarity, implemented in the software *NetStruct* (Greenbaum *et al.* 2016). Unlike model-based approaches, this method does not require pre-specification of a biological model, such as Hardy-Weinberg equilibrium or the number of ancestral populations. We used the spin glass algorithm (Reichardt and Bornholdt 2006) for identifying clusters in the networks in a data-driven manner, and the number of clusters detected is inherent to the method. After generating the genetic similarity matrix, we tested several networks by pruning all edges from the network below a genetic-similarity threshold, with thresholds varying from 0 in increments of 0.01, until 10% of the nodes in the network become isolates (Greenbaum *et al.* 2016). Although clustering with *NetStruct* does not require pre-definition of groups, we tested, post-analysis, whether detected clusters are correlated with the four activity sites, using a Chi-squared test (following Rodger *et al.* 2018). We considered an α-level of 0.01 for determining significant association between genetic cluster compositions and activity sites.

Results

The observed mating system

During the entire recording period (2010–2013), a total of 425 observations of males – either solitary or in groups – were documented. Assuming that individuals of unidentified sex within male-majority groups were males, the total number of observations of males was 1,083. Out of the 425 observations, a substantial number of male groups were of two individuals (50 groups). Therefore, we calculated the proportion of solitary-dominant males out of the total number of males for two scenarios, delineating two extreme estimations of solitary and dominant males: 1) assuming that both males in groups of two males are a dyad of two bachelors; 2) assuming that all males in group size of two are a dyad of solitary-dominant males that interact (as was observed in a few of the identified dominant males; see below). Out of the 1083 observations of males, only 24% to 37% (corresponding to scenarios 1 and 2, respectively) were of solitary males (for per year fractions see Table 1). Given the link between solitary males and dominant ones in this polygynous mating system (Klingel 1998), these findings imply that only a quarter to a third of all males are dominant and have the potential for breeding at each point in time.

The dominance stability of males

A total of 98 individually identified profiles of males were generated: 50 profiles in 2010 and then an additional 8, 34, and 6 profiles during the years 2011, 2012, and 2013, respectively. During the four years of the study, 18 males had highly distinctive marks and were observed more than once (and up to 37 times). Ten of them were observed for more than one year (and up to five years) and were dominant during 2010, 2011 or 2012. Out of these 10 males, eight had remained solitary (or in a group of two, presumably interacting with other dominant males) for a few years – five of them remained solitary for three consecutive years and two males remained solitary for four consecutive years (Fig. 2). Only two of these 10 males were observed in bachelor groups after being observed as solitary. During a breeding season of the same year, all identified males remained in the same social status, i.e., dominant males remained dominant (were observed as solitary), and males

Table 1. The percentage of solitary males out of the total number of male individuals, calculated per year for the two scenarios: dyads are bachelor males, and dyads are solitary males.

		Total	2010	2011	2012	2013	Average ± SD
Dyads are bachelors	No. of all males	1083	338	214	125	406	
	No. of solitary males	264	28	94	40	102	
	% Solitary males	24.4%	8.3%	43.9%	32%	25.1%	27.3% ± 14.9%
Dyads are solitary males	No. of all males	1083	338	214	125	406	
	No. of solitary males	396	61	138	66	131	
	% Solitary males	36.6%	18%	64.5%	52.8%	32.3%	41.9% ± 20.8%

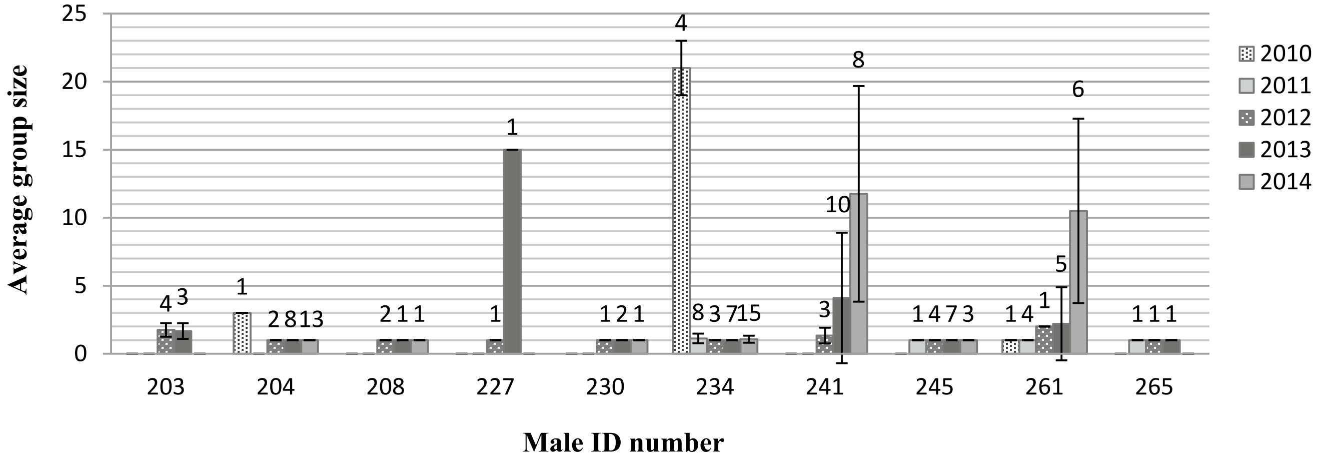

Figure 2. The average group size per year for the 10 individually identified males that were observed for 2–5 years. Error bars represent SD. The number of observations for each male is noted above each bar. Five males were observed solitary for three consecutive years (males #204, #208, #230 #261 and #265) and two males were observed solitary for four consecutive years (males #234 and #245).

in bachelor groups were continuously observed in bachelor groups. In a year in which an identified male was observed in bachelor groups, it was observed in groups of various sizes on different observation days (notice the high variance around the average group size of each identified male, Fig. 2), suggesting that similar to the female groups (Renan *et al.* 2018), bachelors also live in nonstable groups.

During 2012, all 10 males with multi-years observations were observed and were solitary, and their multiple observations in this year sum up to 22. These observations constituted 55% of the total number of solitary male observations of 2012 (n=40, Table 1), implying that these 10 solitary males provide a good representation of the solitary males in the wild ass population.

Population genetic structure

No signs of deviation from HWE proportion in any of the loci were found, besides locus HMS3, which showed significant deviations from HWE proportions ($\chi 2=23.91$, $P < 0.001$). The P_{ID} and the conservative $P_{ID\text{-}sibs}$ were 2.4×10^{-5} and 8.0×10^{-3}, respectively, below the acceptable threshold of $P_{ID\text{-}sibs}$ for wildlife studies ($P_{ID\text{-}sibs}=0.01$; Taberlet & Luikart 1999). Information on the genetic diversity of the population is documented in Renan *et al.* (2015).

F-statistics

Substantial differences in the haplotype frequencies (mtDNA analysis) and in allele frequencies (microsatellite analysis) were observed among the four activity centers (Figs. 3 and 4, respectively). A significant variation among the four activity centers was found in the AMOVA tests on both the mtDNA data and the genomic microsatellite data. In the AMOVA test on the mtDNA of the entire data (n=200) 12% of the variation in the population was explained by the variation among the four sites and the Φ_{PT} estimator was found to be significant ($\Phi_{PT} = 0.12$, $p<0.001$). Pairwise Φ_{PT} comparisons showed a highly significant difference among all sites except between Wadi Ashosh and the Negev Highlands and Wadi Ashosh and the Makhtesh Ramon site (Table 2). Similarly, in the AMOVA test on mtDNA of the unique samples (n=79) the variation among the four sites was found to be significant ($\Phi_{PT} = 0.06$, $p<0.05$). However, pairwise Φ_{PT} comparisons showed significant difference only between Makhtesh Ramon and Wadi Paran (Table S1). In the AMOVA test on the microsatellite data (n=97), both the *FST* estimator and the Φ_{PT} estimator were low but significant (*FST*=0.02, $p<0.05$; $\Phi_{PT}=0.041$, $p<0.001$). The Pairwise *FST* comparisons showed low but significant *FST* values among all sites except between Makhtesh Ramon site and Wadi Ashosh and the Makhtesh Ramon and Wadi Paran (Table S2). The pairwise Φ_{PT} comparisons on the genomic microsatellite data showed stronger differentiation among sites with higher pairwise Φ_{PT} values and higher significance than the pairwise *FST* values (Table 3). In this comparison, all sites, but the Makhtesh Ramon site and Wadi Ashosh, were significantly different. Comparison of the AMOVA results from the mtDNA analysis (unique samples) and the microsatellites analysis revealed low but significant genetic structure in both cases ($\Phi_{PT} = 0.06$, $p<0.05$ and $\Phi_{PT}=0.041$, $p<0.001$, respectively).

Model-based clustering. The STRUCTURE analysis of the genomic microsatellite data could not identify any meaningful structure. The Evanno method (Evanno *et al.* 2005) shows a highest peak of Mean LnP(K) in K=1 (-1412.97); this indicates that the most likely K for the wild ass population is 1 and that there is no genetic structure in the population. When performing a ΔK analysis, there is some weak evidence for K=4 (with a maximal ΔK of 1.74, Table S3, Fig. S1), supporting the presence of four subpopulations. Nevertheless, the ΔK value is relatively low and is a heuristic statistic to which a significance level cannot be assigned, and there was no evidence of partition of individuals among the four subpopulations using their *Q* values (individual membership coefficients) (Fig. S2).

Model-free network-based clustering. In the network represented by the between-individual genetic similarity matrix, without removal of any edges, we detected

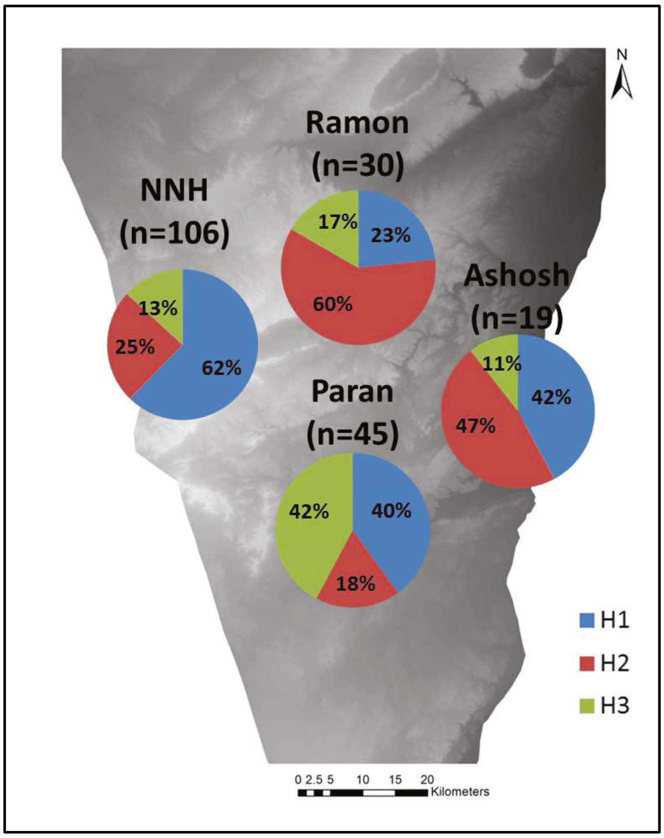

Figure 3. **Mitochondrial haplotype frequencies**. The three haplotype frequencies of the mtDNA marker 12S1 at the four activity centers (entire dataset, n=200). The sample sizes per site are in parentheses next to each site name. "NNH" stands for northern Negev Highlands.

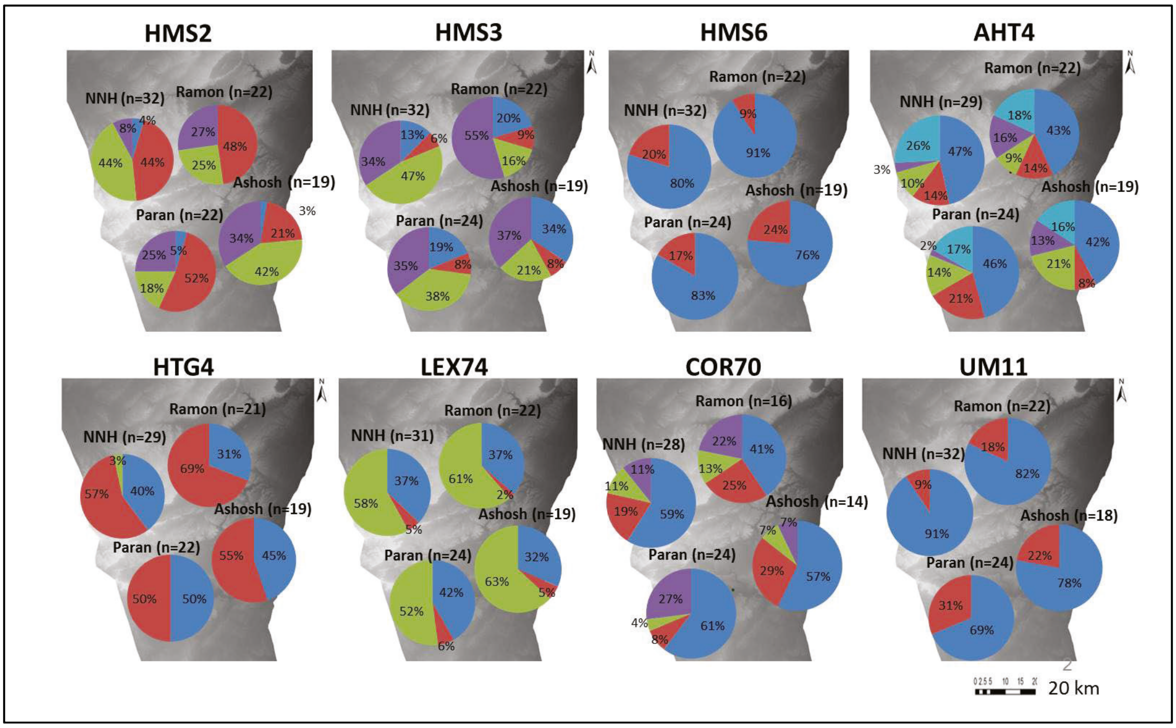

Figure 4. **Allele frequencies**. Allele frequencies of the eight microsatellites loci at the four activity centers (n=97). Each color stands for a specific allele in a specific locus. The sample sizes per locus per site are in parentheses next to each site name. "NNH" stands for northern Negev Highlands.

Table 2. Pairwise Φ_{PT} values (below diagonal) for the mtDNA entire data (n=200) and significance (above diagonal) for each activity center pair. Significant values are indicated with * (P≤0.05), ** (p≤0.01), *** (P≤0.001) or ns (non-significant).

	Wadi Ashosh	Negev Highlands	Wadi Paran	Makhtesh Ramon
Wadi Ashosh	–	ns	*	ns
Negev Highlands	0.051	–	***	***
Wadi Paran	0.099	0.095	–	***
Makhtesh Ramon	0.003	0.189	0.161	–

Table 3. Pairwise Φ_{PT} values for the microsatellites data (below diagonal) and significance (above diagonal) for each activity center pair. Significant values are indicated with * (P≤0.05), ** (p≤0.01) or ns (non-significant).

	Wadi Ashosh	Negev Highlands	Wadi Paran	Makhtesh Ramon
Wadi Ashosh	–	*	**	ns
Negev Highlands	0.039	–	*	*
Wadi Paran	0.061	0.034	–	**
Makhtesh Ramon	0.018	0.042	0.055	–

4 clusters, but these clusters were not significantly associated with the four activity sites (p=0.265, Chi-squared test). When iteratively increasing the edge-pruning threshold, for all networks with edge-pruning thresholds below 0.24, the detected clusters were not significantly associated with the sampling sites, whereas for edge-pruning thresholds above 0.24, all networks were associated with the sites (Fig. 5). Networks with edge-pruning thresholds above 0.28 consisted of less than 90% of the individuals and were not considered in the analysis. These results suggest that the population structure of the four sites is not solid enough to induce sufficiently strong detectable genetic-similarity clusters corresponding to the sites. However, when only strong-similarity edges are included in the analysis, the clusters are significantly associated with the sampling sites, implying that there is a signal of genetic differentiation between the four sites (p=0.000045). The sharp transition in p-value as more edges are removed from the network (from threshold 0.23 to 0.24 in Fig. 5) has also been observed in humans and *Arabidopsis thaliana* (Greenbaum *et al*. 2016, Greenbaum *et al*. 2019) where structure was consistent with geographic partitioning, indicating that the genetic structure we detected here is substantial. In summary, population structure was identified with pre-analysis grouping into the four activity sites. This is supported by both the Φ_{PT} and the F_{ST} values, implying a weak but significant genetic structure.

Discussion

The behavioral polygynous mating system

In the direct observations of Asiatic wild ass individuals, only 24.4% to 36.6% of all male observations were of dominant males. This low proportion of dominant males indicates a polygynous mating system in which only a quarter to a third of all males are potentially involved in the breeding process. Identified males, i.e., those that were recognized individually by their phenotypes – distinguishable natural marking, remained dominant within seasons and over the years, with a dominance tenure of up to at least four years. Because the generation length of Asiatic wild ass is approximately seven years (Saltz & Rubenstein 1995), these long tenures constitute a substantial portion of the reproductive period of these males, implying little turnover of male dominance. Consequently, the estimated proportion of dominant males, which represents dominancy at a given point in time, should be close to the proportion of males that are potentially involved in breeding in each generation.

These findings of behaviorally strong polygyny (Emlen & Oring 1977) are in accord with a previous genetic study of the Negev wild ass population, which found that, genetically, less than 25% of the males contribute to the gene pool each generation (Renan *et al.* 2015) and another genetic analysis indicating 19.5% of males contribute to the gene pool (Greenbaum *et al*. 2018). While the level of polygyny derived from observations (24–36%) and the one derived from genetic analysis (<25%, 19.5%) are similar, the slightly higher behavioral polygyny levels could have several explanations: (1) Not all behaviorally dominant males reproduce (Saltz and Rubenstein 1995); (2) There is variance in the reproductive success of dominant males; (3) Observation bias, resulting from the monitoring being conducted near the main water site in the region, in which the visit rate of dominant and non-dominant males may be unequal. Because water sites can attract dominant males not only as water sources, but also for social interactions (e.g., male-male interactions, dominance advertisement, and for attracting females to their territory), the arrival rate of dominant males to these sites could be higher than that of non-dominant males, inflating the estimation of the fraction of dominant males in the population. The lower estimate of the genetic analysis also suggests that mating of "sneaker" males from bachelor groups, reported to occur in equids (Bowling & Touchberry 1990; Feh 2005), is not a common phenomenon in the wild ass population of the Negev, otherwise we would observe the genetic estimates of polygyny levels to be higher than the behavioral estimate.

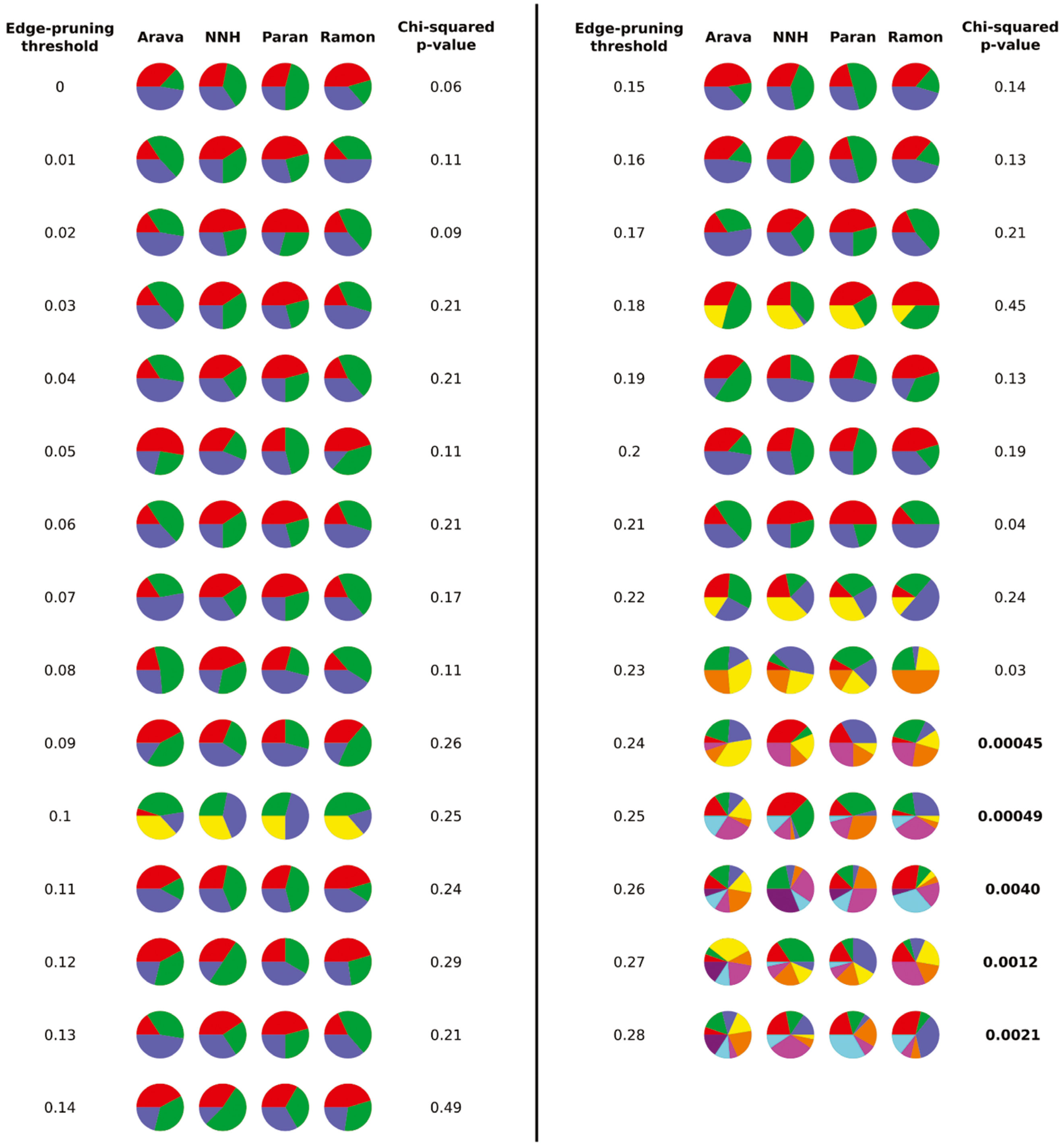

Figure 5. Cluster assignment composition in the four activity sites, generated by network-based genetic clustering. The analysis was conducted on 29 networks, each with a different edge-pruning threshold that was applied for the removal of edges from the between-individual genetic-similarity network (i.e., networks with higher edge-pruning thresholds represent networks including only higher genetic-similarity relations). For each network, assignment of individuals to clusters was determined using network-based clustering, with the number of clusters derived from the data. Each pie-chart describes the composition of cluster assignments in each activity site, where each color represents a single cluster (the number of colors corresponds to the number of detected clusters). For each network, a Chi-squared test was performed to test whether the cluster-assignment compositions are associated with the activity sites. Values in bold indicate significance at an α-level of 0.01.

Population structure

A weak fine-scale genetic structure was detected in the Asiatic wild ass population of the Negev, associated with the four activity centers.

Using F-statistics, similar levels of genetic differentiation among the four activity centers were observed in the mitochondrial analysis and in the microsatellites analysis. The overall Φ_{PT} value was slightly higher in the mtDNA analysis than in the microsatellites analysis. In contrast, pairwise genetic structure inferred from the microsatellites was slightly stronger, as reflected by more significant pairwise comparisons (All microsatellite Φ_{PT} pairwise comparisons were significantly differentiated except between Wadi Ashosh and Machtesh Ramon; Tables 3 vs. Table 2 and S1, see further discussion below).

The model-based approach analysis (*STRUCTURE analysis*) did not reveal any significant population genetic differentiation (Fig. S2). These results were obtained even though the usage of the LOC-PRIOR model implemented in STRUCTURE, which gives a higher prior weight on clustering outcomes that are correlated with the sampling locations, and by doing so, it should allow the detection of population structure even at low levels of divergence (Hubisz *et al.* 2009). The model-free network-based approach (*Netstruct analysis*) did identify clusters that strongly correspond to the four sampling sites when only strong genetic similarities were included in the networks. This cluster composition of the four sites was significantly associated with the activity centers (Fig. 5). The presence of genetic structure within the reintroduced Asiatic wild ass population in the Negev desert is supported by other studies (Gueta *et al.* 2014, Zecerle *et al.* 2020).

Population structuring of such a large mammal in a rather small distribution range, only a few generations after the reintroduction onset is interesting. The genetic structure observed in the wild population could be a remnant of several strong founder events which occurred during the population range expansion, affected also by landscape features, and led to a significant differentiation among sites due to founder effect and drift (Gueta *et al.* 2014). Theoretical studies have demonstrated how during the range expansion of a population, genetic structure can arise within a few generations (Excoffier & Ray 2008; Excoffier *et al.* 2009), and some studies have demonstrated this phenomenon empirically (e.g., Ramakrishnan *et al.* 2010; Short & Petren 2011; Neuwald & Templeton 2013; Gueta *et al.* 2014). If initial genetic differentiations between the four sites were strong, this historic genetic structure may have been preserved even under subsequent gene flow (Hutchison & Templeton 1999; Ramstad *et al.* 2004).

Alternatively, the genetic signature of population structure could also be a result of the species' social behavior. The F-statistics analysis shows slightly higher Φ_{PT} values in the mitochondrial analysis compared to the microsatellites analysis (Φ_{PT} = 0.06, p<0.05, and Φ_{PT} =0.041, p<0.001 respectively), which could suggest stronger structuring in females than in males. However, the comparison between these two results is not straightforward, because differences in sample sizes (79 with mtDNA vs. 97 with microsatellites), number of markers (1 vs. 8), and impacts of genetic drift in haploid and diploid systems confound comparison between uni-parentally and bi-parentally inherited markers (Prugnolle & De Meeus 2002). Nevertheless, the slightly stronger population structure obtained in the mitochondrial data may indicate that females have high fidelity to the water sources, leading to limited gene flow among activity centers. This finding supports previous observations of high water-fidelity of females during the foaling season (Saltz *et al.* 2000), which coincides with the breeding season (Zecherle *et al.* 2020).

The genetic structure in the nuclear DNA (obtained from the microsatellites analysis) suggests that males are also contributing to the limited gene flow among sites (Zecherle *et al.* 2020). The strong polygynous mating system observed in this population suggests that only a small portion of males participate in the breeding process. The results from the multi-year observations imply that these males have long dominance tenure. Moreover, as the Negev population exhibits a fission-fusion social structure (Renan *et al.* 2018), the dominant males are likely to protect a territory (Saltz *et al.* 2000; Neumann-Denzau & Denzau 2007). Territoriality creates strong site fidelity of the dominant males who are the main contributors to the gene-pool for several years (Giotto *et al.* 2016). This combination of male's behavioral traits and females' fidelity to water sources may add to the development of fine-scale genetic structure in the Asiatic wild ass population of the Negev.

If the genetic structure observed in the wild population is a remnant of historic strong founder events, we should expect to see a reduction in the structure signal along the years (Broquet & Petit 2009), however, if the genetic structure is a result of current limited gene flow among sites, the structure signal is expected to remain or even become stronger over time. In the latter case, future studies could examine how this genetic structure is reflected in differences in individuals' diets, and consequently in their microbiomes, within subpopulations across the water sources. Genetic analyses of fecal samples collected in these areas could also be used to address such questions (Paul *et al.* 2023).

Conservation implications and management recommendations

Polygynous mating could provide species an advantage in terms of the evolutionary process, because it increases selection pressures through female preference for high-quality males (Kirkpatrick & Ryan 1991; Birkhead & Parker 1997). However, in a small, reintroduced population, this polygyny could become a disadvantage, as it increases genetic drift and induces erosion of genetic diversity (Nunney 1993). In the reintroduced population of Asiatic wild ass in the Negev, where low levels of genetic diversity have been previously documented (Renan *et al.* 2015), accelerated rates of genetic diversity loss due to high levels of polygyny can increase the risks to the population's long-term persistence (Nunney & Campbell 1993; Newman & Pilson 1997).

Furthermore, if the genetic structure observed in the reintroduced population is an outcome of the current limited gene flow among sites, this population subdivision can affect the genetic diversity of the populations in several ways. On the one hand, if the subpopulations have low effective sizes, accelerated drift and inbreeding are expected to dramatically reduce the genetic variation of each subpopulation, increasing the risks from inbreeding in each of these subpopulations. On the other hand, fine-scale population structure increases the variance effective size and reduces the rate of loss of genetic diversity in the overall reintroduced population, because each subpopulation may preserve unique genetic variations that might have been lost in the case of a panmictic population (Chesser *et al.* 1993; Sugg & Chesser 1994; Sugg *et al.* 1996; Neuwald and Templeton 2013; Templeton 2025 in preparation). Monitoring the genetic compositions of each subpopulation over time will enable evaluating the two potential effects of population structuring on the genetic diversity in the population.

This study on the wild ass mating system and the population genetic structure in the Negev population can inform management decisions regarding connectivity between activity sites. For example, if future studies demonstrate that genetic structuring is increasing in the population, and that this increase is associated with a substantial loss of genetic diversity at the local subpopulation level, active management for increasing landscape connectivity among subpopulations should be considered (Kaczensky *et al.* 2011). Since all activity centers were created around permanent water sources, adding more water sources between the activity centers may increase connectivity between sites as well as population effective sizes (Greenbaum *et al.* 2018).

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Appendix 1

PCR conditions for locus12S1:

DNA extracts were diluted 1:10 for use as a template in PCR reactions, and reactions were carried out on a T Professional basic thermal cycler (Biometra) in a volume of 20 µl containing 3 µl of DNA template, 1xTaq Rxn Buffer, 2 mM MgSO4 , 0.5 µM of each primer, 0.125 mM of each dNTP and 1 unit of Taq polymerase (Hy-Labs, Israel) with the following thermal cycles: 94 °C for 5 min; 40 cycles of 94 °C for 15s, 62 °C for 20s and 72 °C for 12s; 72 °C for 5 min.

Table S1. Pairwise Φ_{PT} values (below diagonal) for the mtDNA data of unique samples (N=79) and significance (above diagonal) for each activity center pair. Significant values are indicated with * (P≤0.05), ** (p≤0.01), *** (P≤0.001) or ns (non-significant). Sample size in brackets.

	Wadi Ashosh	Negev Highlands	Wadi Paran	Makhtesh Ramon
Wadi Ashosh (15)	–	ns	ns	ns
Negev Highlands (24)	0.000	–	ns	ns
Wadi Paran (21)	0.071	0.000	–	**
Makhtesh Ramon	0.000	0.086	0.201	–

Table S2. Pairwise F_{ST} values for the microsatellites data (below diagonal) and significance (above diagonal) for each activity center pair. Significant values are indicated with * (P≤0.05), ** (p≤0.01) or ns (non-significant).

	Wadi Ashosh	Negev Highlands	Wadi Paran	Makhtesh Ramon
Wadi Ashosh	–	*	*	ns
Negev Highlands	0.023	–	*	*
Wadi Paran	0.013	0.015	–	ns
Makhtesh Ramon	0.011	0.025	0.012	–

Table S3. The Evanno table output for all tested K. The highest ΔK obtained for k=4.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln′(K)	|Ln″(K)|	ΔK
1	20	–1412.965	0.623467	–	–	–
2	20	–1474.300	59.351345	–61.335	32.505	0.547671
3	20	–1503.130	59.401932	–28.830	84.985	1.430677
4	20	–1446.975	33.982393	56.155	59.000	1.736193
5	20	–1449.820	34.210888	–2.845	24.565	0.718046
6	20	–1428.100	18.865368	21.720	13.515	0.716392
7	20	–1419.895	9.000905	8.205	–	–

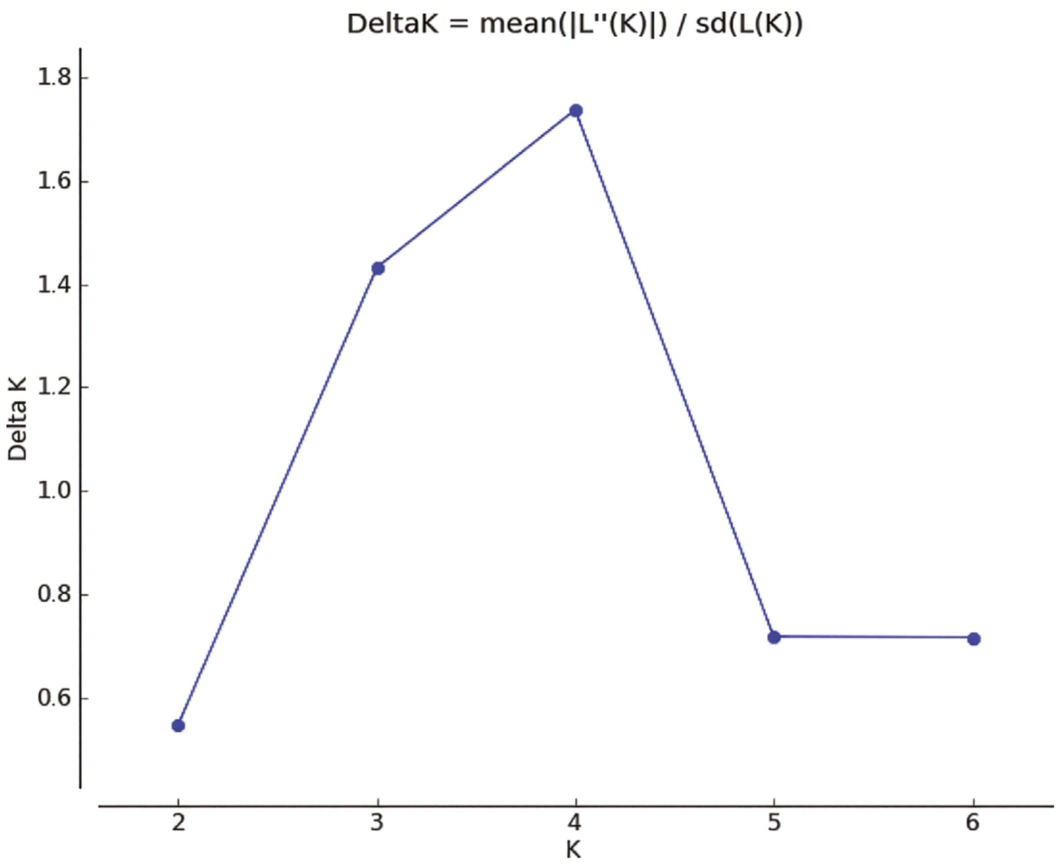

Figure S1. Δ*K* (the second-order rate of change of K) values as a function of *K* (the number of genetic clusters). According to the Evanno method, the highest Δ*K* is a good predictor of the real number of clusters in the population.

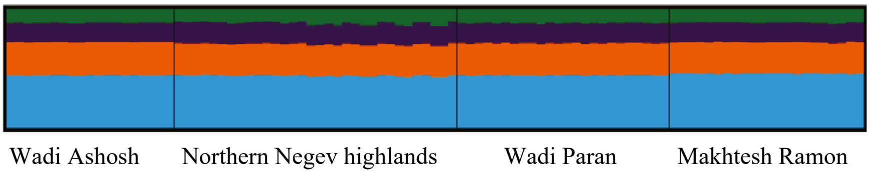

Figure S2. Individual membership coefficients (*Q*) for the four clusters identified by STRUCTURE (*K*=4). Each individual is represented by a single vertical line. The sampling location for each sampled individual is indicated below as one of the main activity centers. The graph presents the main cluster obtained after clumping.