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Stochastic modelling of shifts in allele frequencies reveals a strongly polygynous mating system in the re-introduced Asiatic wild ass

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Abstract

Small populations are prone to loss of genetic variation and hence to a reduction in their evolutionary potential. Therefore, studying the mating system of small populations and its potential effects on genetic drift and genetic diversity is of high importance for their viability assessments. The traditional method for studying genetic mating systems is paternity analysis. Yet, as small populations are often rare and elusive, the genetic data required for paternity analysis are frequently unavailable. The endangered Asiatic wild ass (*Equus hemionus*), like all equids, displays a behaviourally polygynous mating system; however, the level of polygyny has never been measured genetically in wild equids. Combining noninvasive genetic data with stochastic modelling of shifts in allele frequencies, we developed an alternative approach to paternity analysis for studying the genetic mating system of the re-introduced Asiatic wild ass in the Negev Desert, Israel. We compared the shifts in allele frequencies (as a measure of genetic drift) that have occurred in the wild ass population since re-introduction onset to simulated scenarios under different proportions of mating males. We revealed a strongly polygynous mating system in which less than 25% of all males participate in the mating process each generation. This strongly polygynous mating system and its potential effect on the re-introduced population's genetic diversity could have significant consequences for the long-term persistence of the population in the Negev. The stochastic modelling approach and the use of allele-frequency shifts can be further applied to systems that are affected by genetic drift and for which genetic data are limited.

Keywords: allele-frequency shifts, conservation genetics, elusive species, *Equus hemionus*, genetic drift, paternity analysis

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Introduction

Genetic variation represents the adaptive potential of a population and, therefore, may influence the population's long-term viability and sustainability (Hughes *et al.* 2008). The mating system of a species can strongly affect the genetic diversity of populations through its effect on the proportion of breeding individuals (reviewed in Sugg *et al.* 1996 and Storz 1999). For example, in polygynous and polygynandrous mating systems, in which not all individuals have an opportunity to mate, the proportion of individuals contributing their genes to the gene pool is considerably low. This reduces the variance effective size (N_{ev}), accelerates the effects

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of genetic drift and, consequently, increases the rate at which genetic diversity is lost from the population. This effect has been shown both theoretically (Templeton 2006) and empirically (Pope 1992; Sugg *et al.* 1996; Balloux *et al.* 1998). Therefore, studying mating systems and their impacts on populations' genetic diversity is of theoretical importance for behavioural ecology and population genetics and has potential consequences for conservation genetics and management plans (Anthony & Blumstein 2000). This is particularly the case for small populations, as they are prone to extensive loss of genetic variation due to genetic drift (Templeton 2006).

Direct observations are the traditional method for detecting species' mating systems. However, as cryptic reproduction is a common phenomenon in vertebrates (Parker & Waite 1997), the genetic mating system (i.e. the actual proportions of breeding individuals that contribute their genes to the gene pool) cannot always be inferred by observing social structure patterns (Griffith *et al.* 2002). As molecular techniques improve, they have been increasingly used to reveal the actual genetic mating system of species (Hughes 1998). The most common method that uses molecular data for studying the genetic mating system is parentage analysis, in which the DNA fingerprinting of the parents can be detected by examining the offspring's DNA and the potential parents' DNA (reviewed in Jones *et al.* 2010). For this analysis, good sample coverage of the population is needed; preferably, at least one definite parent–offspring link should be known, and ideally, 10 loci or more are needed (Nielsen *et al.* 2001; Jones & Ardren 2003). In the study of small populations (e.g. threatened or re-introduced populations), the populations are often elusive and direct observations are rare; thus, detecting parent–offspring links is difficult. Moreover, as small populations are at risk and researchers make an effort to avoid unnecessary disturbances, good sample coverage of high-quality samples obtained from tissue or blood is usually rare. To overcome these limitations and to obtain sufficient sample coverage for elusive populations, researchers commonly use noninvasive genetic sampling by collecting shed hair or faeces as the DNA source (Beja-Pereira *et al.* 2009). However, the low quality and quantity of DNA obtained by noninvasive sampling is often not sufficient for amplifying enough loci for reliable parental analysis (but see Morin *et al.* 1994; Gagneux *et al.* 1999; Gerloff *et al.* 1999; Constable *et al.* 2001). Hence, for small and elusive populations, for which studying the mating system and its potential impact on genetic diversity is of particular importance, the data required for paternity analysis are often not available.

The Asiatic wild ass (*Equus hemionus*) is an elusive endangered species (Moehlman *et al.* 2008). The species was once abundant in western Asia, including the Negev Desert of Israel, but declined throughout its range due to hunting and habitat loss and eventually became extinct in Israel. Between 1982 and 1993, 38 Asiatic wild asses were re-introduced from a breeding core to the Negev Desert, Israel. In 1991, during the re-introduction period, a genetic survey of all animals in the breeding core (hereafter referred to as the 'breeding core population') was conducted, and their blood samples were preserved (Sinai 1994). The current population in Israel, in the Negev Desert and the Arava Valley, is estimated at more than 250 individuals.

Direct observations on the re-introduced population in Makhtesh Ramon in the years that followed the re-introduction indicated that the Asiatic wild ass follows a fission–fusion social structure (Saltz *et al.* 2000). This social structure is characterized by resource-defence polygyny (Klingel 1975; Rubenstein 1994) in which solitary males are considered to be the dominant territorial males obtaining most of the mating opportunities. In a recent study, based on direct observations on the current wild ass population in the northern Negev Highlands (the most populated activity centre), 27% of all males' observations were of solitary males (Renan 2014). If this relatively small proportion of solitary males has a long dominance tenure and this observed mating system reflects the genetic mating system, this combination could have critical consequences for the genetic diversity of the re-introduced population and for its long-term persistence.

Because the real level of polygyny has never been measured genetically in the wild ass or in any wild population of equids, in this study, we aimed to explore the genetic mating system of the re-introduced Asiatic wild ass in the Negev Desert. As an alternative approach to paternity analysis, we applied simulations of a stochastic model of allele frequencies. The study approach was based on the fact that genetic drift is a stochastic process affected by the proportion of males contributing to the gene pool in each generation. Therefore, by comparing the strength of the genetic drift that occurred from the founding to the current population to the drift that occurred in simulated populations under different proportions of mating males, an estimation of the proportion of males contributing to the gene pool—the genetic mating system—could be obtained.

Materials and methods

The re-introduced population

The Asiatic wild ass population in Israel was re-introduced from a breeding core established in 1968 at the

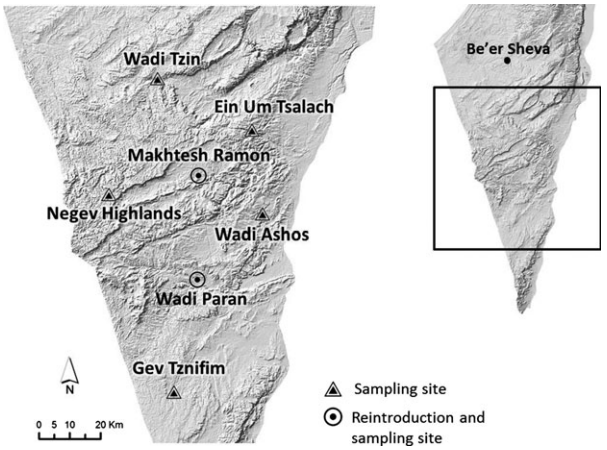

Fig. 1 The seven sampling sites in the wild ass distribution range.

Hai-Bar Yotvata Reserve from 11 individuals belonging to the Iranian (*E. h. onager*, 3 M, 3 F) and the Turkmenian (*E. h. kulan*, 2 M, 3 F) subspecies. Between 1982 and 1987, the Israel Nature and Parks Authority (INPA) re-introduced 28 individuals (14 M, 14 F) at Ein Saharonim, Makhtesh Ramon (Saltz & Rubenstein 1995). Between 1992 and 1993, another 10 individuals (3 M, 7 F) were re-introduced to Wadi Paran. Both populations were intensively monitored in the years following release (Sinai 1994; Saltz *et al.* 2006) and routinely monitored thereafter. During the 1990s, the wild ass population naturally expanded its geographical range to the northern Negev Highlands and to the Arava Valley (Fig. 1), and no severe population reduction was documented during this period. We refer to the current wild population as the 'wild population'.

Sample collection, DNA extraction and microsatellite genotyping

We sampled 219 faecal samples, 11 blood samples and 12 tissue samples (road kills and GPS-collared individuals) from the wild population. The faecal samples were collected from seven sites distributed throughout the distribution range of the Asiatic wild ass population (Makhtesh Ramon, Wadi Paran, Wadi Ashosh, northern Negev Highlands, Ein Um Tsalach, Wadi Tzin and Gev Tznifim; Fig. 1), from September 2012 to October 2013. We collected only fresh faeces within two hours after sunrise to minimize exposure to high temperatures, which accelerates DNA degradation (Nsubuga *et al.* 2004). To increase the amplification efficiency of the samples, only the outer layer of the faeces was removed, using cotton swabs, and stored on ice immediately after collection (Renan *et al.* 2012). The samples were transferred to a freezer (−20 °C) within a few hours after collection and extracted the following day.

The 'breeding core population' included the 31 blood samples (13 F, 18 M) that were taken in 1991 from the Asiatic wild ass breeding core (Sinai 1994) and preserved since then at −80 °C.

DNA extraction from blood and tissue samples was performed using a QIAamp DNA Mini Kit (QIAGEN, Cat. No. 51304) following the manufacturer's instructions. Faecal samples were extracted according to the QIAamp® DNA Stool Mini Kit (QIAGEN, Cat. No. 51504) protocol with modifications as described by Renan *et al.* (2012). Of 24 microsatellite loci that were tested, only eight (HMS2, HMS3, HMS6, AHT4, HTG4, LEX74, COR070 and UM11) showed sufficient amplification success, low genotyping error and polymorphism. These loci were used for amplification and genotyping (for references and protocols, see Appendix S1, Supporting information).

Genetic variability analysis

For both the wild population and the breeding core population, data for each locus were tested for linkage disequilibrium and for deviations from the Hardy–Weinberg equilibrium (HWE) using ARLEQUIN version 3.5.1.3 (Excoffier & Lischer 2010). The loci were tested for scoring errors due to stuttering, for allelic dropout and for the presence of null alleles, using MICRO-CHECKER version 2.2.3 (van Oosterhout *et al.* 2004). For each locus, GENALEX 6.41 (Peakall & Smouse 2006) was used to estimate the number of alleles (*NA*), the 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.

To assess whether a significant reduction in the genetic diversity has occurred since re-introduction, the levels of differences in the number of alleles and in the H_E between the breeding core and the wild populations were tested using a one-tailed Wilcoxon's signed-ranks test (Maudet *et al.* 2002). The genetic difference between the two populations was tested using an AMOVA test (Analysis of Molecular Variance) with a *Codom-Allelic* distance option implemented in GENALEX 6.41 (Peakall & Smouse 2006), and the F_{ST} value was calculated based on the number of different alleles as determined by ARLEQUIN version 3.5.1.3 (Excoffier & Lischer 2010). The significance of the F_{ST} value was calculated using permutation tests. For these comparisons, only samples with information on at least seven of the eight microsatellites were

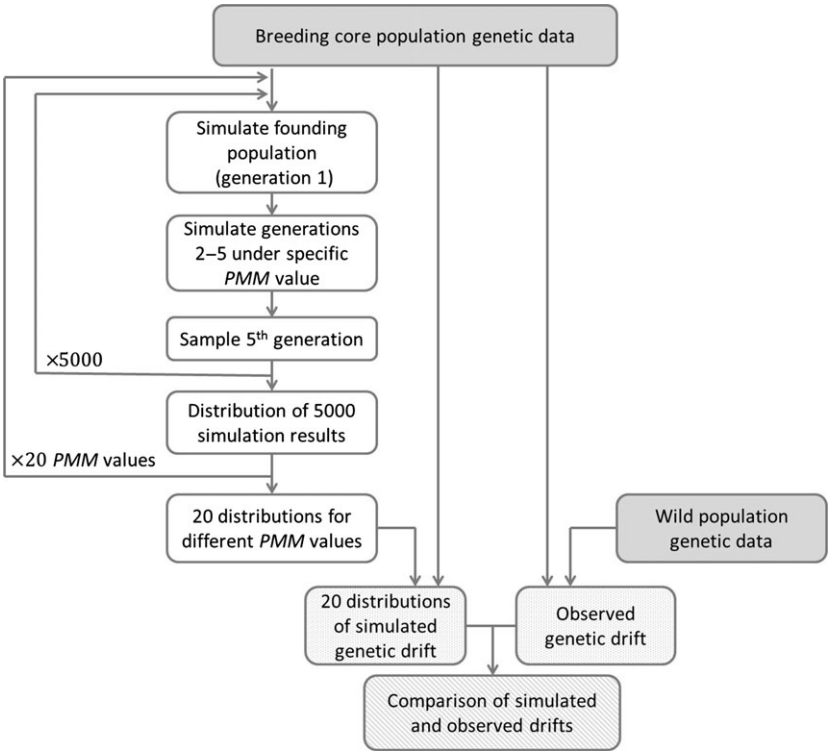

Fig. 2 Schematic outline of the stochastic model and analysis approach. The genetic mating system of the wild ass population was examined using simulations under different proportions of mating males (*PMM*), and comparisons of the simulated and observed genetic drift measures.

included in the analyses (breeding core population, $n = 27$; wild population, $n = 104$).

Stochastic model

The genetic mating system of the Asiatic wild ass was explored using a stochastic, individual-based model. As equid males are partitioned into two classes, dominant males and bachelor males, the genetic dynamics were modelled under different scenarios of proportions of mating males (henceforth denoted '*PMM*'), following Wade & Shuster (2004). The model simulated a founding population and four subsequent generations, which is equivalent to the time that has passed since re-introduction onset (the wild ass's generation length is approximately 7 years, Saltz & Rubenstein 1995). The last simulated generations were sampled, and the resulting distributions of genetic drift measurements were compared with those of the current wild ass population (Fig. 2).

We accounted for population demography in terms of the number and sex of adult individuals in each generation according to the known numbers of surviving adult individuals from the re-introduction follow-up studies and the R_0 from the wild ass life-history table (for the demographic history, see Appendix S2 and Table B1, Supporting information). The model tracks only adults at their reproductive stage, as individuals that do not survive to this stage do not affect the genetic composition of the population in following generations. Each simulated individual was characterized by a genotype for the eight microsatellite markers. In the founding population (first generation), the individual genotypes were generated using the gene pool of the breeding core population, assuming that it provides a good representation of the allele frequencies of the Makhtesh Ramon founders. These genotypes were simulated by randomly selecting alleles according to the allele frequencies of the breeding core gene pool.

From the individuals of the founding population, a 'mating males' group was defined, consisting of a random selection of a fixed *PMM* out of the total males. Then, the genotype of each individual in the next generation was generated by randomly choosing (with replacement) one female from the overall pool of females and one male from the 'mating male' group of the previous generation, and selecting, for each locus, one allele from each parent. This procedure imposed a variance in lifetime reproductive success approximated by a Poisson distribution (Nunney 1993) for both females and mating males. The following generations were generated in a similar manner, keeping the *PMM* value constant throughout the simulation, and redefining the 'mating males' group for each generation. The known genotypes of the individuals that were

re-introduced to Wadi Paran were explicitly added to the second generation and not simulated. To avoid sampling bias due to partial sampling, the population of the last generation in each simulation was sampled for each microsatellite locus independently, taking individuals' genotypes from the last generation according to the sample sizes obtained from the wild population (i.e. sample allele frequencies were used rather than population allele frequencies), and all analyses were performed on this sampled fifth generation. Overall, twenty model scenarios were simulated; each had a different *PMM* value (0.05 to 1 in 0.05 increments), and each was simulated 5000 times.

The model included the assumptions that all females have the same potential to reproduce, but that the number of offspring is Poisson-distributed (an assumption supported by data from the Negev wild ass population showing a variance to mean ratio of adult female lifetime reproductive success close to one, D. Saltz, unpublished data). The variance in reproductive success amongst mating males is also assumed to be Poisson-distributed, a robust assumption as it has been shown that this variance has little impact on the total variance, and that most variance is due to the difference between the two classes of mating and nonmating males (Wade & Shuster 2004). Additionally, it is assumed that there is no population spatial structure (i.e. mating between 'mating males' and females is random), and generations were considered discrete (no overlap between generations). The model was developed using MATHEMATICA (Wolfram 1999).

Model analysis

The general approach of the model analysis is similar to the approximate bayesian computation (ABC) approach (Beaumont *et al.* 2002). Unlike a typical application of ABC, much is known about the history of this population by direct observation, so we could simulate the population's history accurately without the need to specify priors on various demographic variables. This simplified our simulations such that all inference could be focused on the single behavioural parameter of interest, *PMM*, thus enhancing computational efficiency. We explored a parameter space of 20 *PMM* values, ranging from 0 to 1 with 0.05 increments, which is equivalent to using a uniform grid of evenly spaced atoms as a prior. Using a uniform grid search over the parameter space, we eliminated insufficiency that can arise from an approximate posterior density of a summary statistic (Nakagome *et al.* 2013). For each *PMM* value, the distribution (generated by the 5000 simulations) of locus-level measures (H_E and *NA*, $n = 8$ markers) and allele-level measures, based on shifts of allele frequencies from the founding population to the sampled fifth generation ($N = 29$ alleles across all loci), were compared with the corresponding measures in the current wild population.

The analyses at the locus-level were performed using two approaches: (i) treating H_E and *NA* in each locus as an independent statistical test; and (ii) evaluating mean H_E and *NA* for all loci. These analyses revealed no information regarding the mating system, and therefore, their methods and results are presented in Appendix S3 (Supporting information).

Despite the possible increase in the statistical power due to the relatively higher number of alleles compared to loci in a given sample, analysis of genetic diversity at the allele-level is not a common practice for the following reason: although genetic drift induces shifts in allele frequencies with a similar force on all alleles, the distributions of shifts in allele frequencies are not comparable amongst different alleles. This is because the variance of these distributions depends on the initial frequency of the allele, as demonstrated by the Wright–Fisher model (Wright 1931). To overcome this issue, two approaches were used to compare observed and simulated shifts in allele frequencies: (i) multiple independent statistical tests of shifts in allele frequencies for different alleles; and (ii) stabilization of the variance of shifts in allele frequencies. Below, we describe both of these approaches in detail.

Multiple independent tests. We used the simulated distributions to generate, for each allele, a credible interval, defined by different α levels (CI_α), of the expected allele frequency of the current wild population. The probability for a *PMM* value was attained by considering each allele as a statistical test (whether or not it falls within the CI_α) and by accounting for all alleles by treating them as a series of statistical tests. However, as allele frequencies within the same locus are not independent random variables (allele frequencies in a locus sum to 1), a procedure of randomly removing one allele from each locus was used to generate a set of independent tests.

For a given α and *PMM*, the overall number of alleles not falling within the CI_α was defined as $A_\alpha = \sum_l a_{\alpha,i}$ where $a_{\alpha,i}$ is the number of alleles in each locus i (overall l loci) in which the observed allele frequency in the wild population did not fall within the CI_α. The probability to observe exactly k alleles not falling within the CI_α after the random removal of one allele from each locus, $Q(k)$, depends on $a_{\alpha,i}$ and the total number of alleles in each locus, b_i:

$$Q(k)=\begin{cases}\prod_{i=1}^{l}\frac{a_{\alpha,i}}{b_i} & k=A_\alpha-l\\ \sum_{\substack{n_1,n_2,\ldots,n_k\\ 1\le n_1,<n_2<\ldots n_{k-A_\alpha+l}\le l}}\ \prod_{i\in\{n_1,n_2,\ldots,n_{k-A_\alpha+l}\}}\left(1-\frac{a_{\alpha,i}}{b_i}\right)\prod_{j\in\{1,2,\ldots,l\}/\{n_1,n_2,\ldots,n_{k-A_\alpha+l}\}}\frac{a_{\alpha,i}}{b_j} & A_\alpha-l+1\le k\le a_\alpha\\ 0 & \text{Otherwise}\end{cases}. \qquad \text{eqn1}$$

We define the total number of alleles as $B=\sum_i^l b_i$, and therefore, the remaining number of alleles after removal is $B-l$. The probability to obtain the observed $a_{\alpha,i}$ or greater numbers can be calculated using the cumulative distribution function (CDF) of a binomial distribution (with probability of success of each trial α and $B-l$ trials). Combining these two procedures allows for the calculation of the probability of obtaining the observed $a_{\alpha,i}$ prior to the allele removal procedure, P_α:

$$P_\alpha=\sum_{k=0}^{B-l}Q(k)\sum_{n=k}^{B-l}\binom{B-l}{n}\alpha^n(1-\alpha)^{B-l-n}. \qquad \text{eqn 2}$$

Hence, P_α can be used as a *P*-value for the rejection of a given *PMM* value hypothesis. By calculating P_α for different α levels (between 0.01 and 0.5), we were able to identify the α that generates the statistical test with the strongest statistical power.

Variance stabilization. In this approach, we removed the dependence of the shifts in the allele frequencies from the initial allele frequencies. Under the Wright–Fisher model, the distribution of allele frequency due to genetic drift, p_{t+1}, can be approximated by a multinomial distribution, with a variance that depends on the frequency of the previous generation, p_t:

$$Variance\,(p_{t+1})=\frac{1}{n}p_t(1-p_t). \qquad \text{eqn 3}$$

This creates dependence of the variance on past allele frequencies. Neuwald & Templeton (2013) have shown that a variance-stabilizing transformation on allele frequencies can be used to compare and average shifts in allele frequencies from different alleles and loci as a way of measuring genetic drift. We used the following stabilizing transformation (Bishop *et al.* 1975)

$$a=\frac{1}{2}\left(arcsin\sqrt{\frac{np}{n+1}}+arcsin\sqrt{\frac{np+1}{n+1}}\right), \qquad \text{eqn 4}$$

to calculate a comparable estimate of genetic drift for each allele under each *PMM* value, with allele frequency p and sample size n. The variance of the resulting transformed distribution for each allele is independent of the initial allele frequency. This transformation was used both on the simulated and observed (wild population) allele frequencies from all loci. Although the sample sizes of the stochastic simulation were large enough to allow the use of the more common variance-stabilizing transformation for multinomial distributions $a=\sqrt{\arcsin(p)}$ suggested by Neuwald & Templeton (2013), the wild population sample size was small, and therefore, the transformation presented in eqn 4, which takes into account sample size, is more appropriate.

The frequency shift in the variance-stabilized allele frequencies can be used to define a measure of genetic drift:

$$\Delta a=a_t-a_0, \qquad \text{eqn 5}$$

where a_t (t = 5) is either the simulated allele frequency in the fifth generation or the observed allele frequency in the current wild population, and a_0 is the founding population allele frequency in the same allele. This measure can be calculated over all alleles and all simulations for a given *PMM* value as an estimate of the force of genetic drift during the time period in question. We calculated Δa for each allele in the wild population and 5000 simulated Δa values for each allele in each *PMM* scenario. This allowed us to compare the distribution of frequency-shift values from the wild population to the 20 simulated distributions of each *PMM* value using a Kolmogorov–Smirnov test as a goodness-of-fit analysis.

A two-sample Kolmogorov–Smirnov test allows for a comparison of two empirical probability distributions assuming the samples are independent. However, as pointed out earlier, for a locus with m alleles (and m allele frequencies), only a selection of $m-1$ of them is independent, and the same applies for the Δa values. To ensure the independence of the Δa values, simulations were used. For each *PMM* value, 1000 two-sample Kolmogorov–Smirnov tests were conducted, in which, for each test, one Δa value was removed randomly from each locus (resulting in 21 Δa values of the 29 alleles), both from the observed and simulated data. Each test compared an empirical distribution of 105 000 simulated drift values (5000 simulation results for 21 alleles) with an empirical distribution of 21 drift values of the current wild population. The resulting *P*-value and test statistics results were averaged over the 1000 two-sam-

ple Kolmogorov–Smirnov tests for each *PMM* value. For applicability considerations of the variance stabilization approach, see Appendix S4 (Supporting information).

For both the multiple independent tests approach and the variance stabilization approach, sensitivity analyses concerning the model assumptions were conducted for the population growth rate (±10%), variance in female reproductive success (minimal possible variance for an underdispersed distribution and twice the variance of a Poisson distribution for an overdispersed distribution) and the male–female sex ratio (6:4 and 4:6 male to female ratios).

Results

Microsatellite genotyping

Most of the breeding core population samples (27/31) were successfully amplified by at least seven microsatellite loci, and sample size per locus ranged from 26 to 30 (Table 1). In the wild population, all blood and tissue samples were successfully amplified for genotyping in at least seven microsatellite loci. However, due to the difficulties of extracting and amplifying the noninvasive faecal samples of the wild population, the sample size per locus in the wild population ranged from 88 to 127 samples (Table 1) of 219 samples collected (40-58%). Details of sampled genotypes are presented in Appendix S1 (Supporting information).

Genetic variability

In the breeding core population, no significant deviations from HWE proportions in any of the loci were found. In the wild population, only the locus HMS3 showed significant deviations from HWE proportions (χ^2=23.91, $P < 0.001$). The mean number of alleles per locus (*NA*) was 3.6 in the breeding core population and 3.4 in the wild population (Table 1). Most alleles (21/23) of the breeding core population were present in the wild population (Fig. 3). The mean unbiased expected heterozygosity (H_E) was 0.56 in the breeding core population and 0.54 in the wild population (Table 1). No significant difference was found in the mean *NA* or in the H_E between the two populations (one-tailed Wilcoxon's signed-ranks test). Nevertheless, in most loci, a substantial shift in allele frequencies occurred from the breeding core to the wild population (Fig. 3), leading to a significant difference between the two populations (AMOVA test: F_{ST} = 0.058, $P < 0.001$).

Model analysis of genetic diversity measures and shifts in allele frequencies

In contrast to the analyses of the locus-level measures of H_E and *NA* (see Appendix S3, Supporting information) that did not allow for a useful examination of the genetic mating system due to a lack of statistical power, the analysis at the allele-level was able to provide meaningful information on the proportion of mating

Table 1 Characterization of the eight microsatellite loci of the breeding core and wild populations of Asiatic wild ass

Population	Locus	*N*	*NA*	Effective alleles	H_O	H_E	Inbreeding coefficient
Breeding core population	HMS2	28	4	2.52	0.79	0.60	−0.30
	HMS3	28	4	3.41	0.57	0.71	0.19
	HMS6	30	2	1.60	0.43	0.38	−0.16
	AHT4	27	7	4.70	0.70	0.79	0.11
	HTG4	28	3	2.12	0.50	0.53	0.05
	LEX74	29	3	2.56	0.69	0.61	−0.13
	COR70	26	4	3.87	0.85	0.74	−0.14
	UM11	27	2	1.12	0.11	0.10	−0.06
	Mean	27.9	3.6	2.74	0.58	0.56	−0.05
Wild population	HMS2	113	4	2.97	0.70	0.66	−0.05
	HMS3	125	4	3.18	0.65	0.69	0.06
	HMS6	127	2	1.44	0.36	0.31	−0.18
	AHT4	113	5	3.28	0.71	0.70	−0.02
	HTG4	109	3	2.01	0.57	0.50	−0.13
	LEX74	123	3	2.17	0.62	0.54	−0.15
	COR70	88	4	2.53	0.61	0.61	−0.01
	UM11	120	2	1.44	0.28	0.30	0.10
	Mean	114.8	3.4	2.38	0.56	0.54	−0.05

NA, number of alleles; Ho, observed heterozygosity; H_E, unbiased expected heterozygosity.

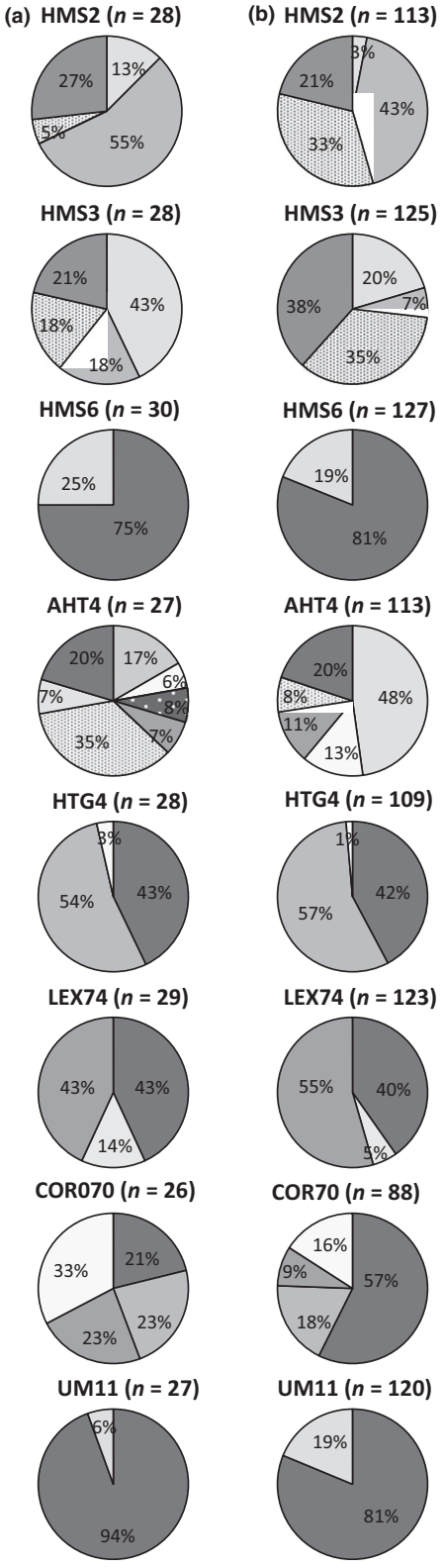

Fig. 3 Allele frequencies of the eight microsatellite loci of the breeding core population (a) and the wild population (b). Changes in the allele frequencies occurred in all loci; two alleles of AHT4 were lost from the breeding core population.

males. The multiple independent tests approach rejected various *PMM* models, narrowing the possible range of mating systems. When calculating the probability for obtaining the observed number of alleles (from all loci) that do not fall within the CI_α for each of the different α-levels (Table B2 in Appendix S2, Supporting information), the most powerful test, in which most *PMM* models could be rejected, was obtained for $\alpha = 0.26$. The number of alleles that were outside the $CI_{0.26}$ in each of the *PMM* values and the probability for obtaining this number are presented in Table 2. For example, in locus HMS3, two alleles did not fall within the $CI_{0.26}$ at *PMM*=0.15; one of these alleles is allele 151, which did not fall within the *CI* from the $CI_{0.26}$for all *PMM* models above 0.15 (Fig. 4). Under $\alpha = 0.26$, the hypothesis of $PMM \geq 0.25$ was rejected with $P < 0.05$; the hypothesis of $PMM \geq 0.4$ was rejected with $P < 0.01$, and the hypothesis of $PMM \geq 0.55$ was rejected with $P < 0.001$ (Table 2, last row). These results indicate a genetic mating system in which less than 25% of the males participate in the mating process in each generation. This result was robust, as indicated by the sensitivity analyses (Appendix S2, Supporting information).

Following the variance stabilization approach, the simulated distributions of shifts in allele frequencies showed different dispersions for different *PMM* models (Fig. 5). As expected, for scenarios with lower *PMM* values, the shifts in the transformed allele frequencies (Δa values, eqn 5) were notably more dispersed, indicating stronger genetic drift. The *P*-values obtained by the Kolmogorov–Smirnov tests were not low enough to reject any of the *PMM* models (Fig. 6). Nevertheless, the goodness-of-fit analysis, which compared the frequency-shifts distribution of the wild population with each of the distributions of the simulated *PMM* values, showed a distinct maximal fit for the *PMM* value of 0.1 (Fig. 6). This implies that a genetic mating system in which 10% of the males contribute their genes to the gene pool represents the best fit between the simulated and the observed genetic data. This result was supported by the sensitivity analyses conducted (Appendix S2, Supporting information).

Discussion

Studying the genetic mating system of species and its effect on the species' genetic diversity has become a common practice in the fields of animal behaviour and conservation genetics (Constable *et al.* 2001; Storz *et al.* 2001; Dobson *et al.* 2004; Wright *et al.* 2012). Yet, methods for revealing the genetic mating system of elusive species are lacking. The wild ass population of the Negev has experienced significant genetic drift since

Table 2 Number of alleles in each loci whose shift in allele frequency did not fall within the credible interval ($a_{1\text{-}8}$) for each *PMM* model, defined by the most informative α-level ($\alpha = 0.26$). The *P*-value for the rejection of the hypothesis of a given *PMM* model was calculated using eqn 2.

	PMM																			
	0.05	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5	0.55	0.6	0.65	0.7	0.75	0.8	0.85	0.9	0.95	1
a_1 (HMS3)	0	0	2	2	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4
a_2 (HMS2)	1	1	1	1	1	2	2	2	2	3	3	3	3	3	3	3	3	3	3	3
a_3 (AHT4)	1	2	2	2	2	4	4	5	5	5	5	5	5	5	5	5	5	5	5	5
a_4 (HTG4)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a_5 (Lex74)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	2	1	1
a_6 (HMS6)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a_7 (Cor70)	1	1	1	1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
a_8 (Um11)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
P-value	0.894	0.788	0.525	0.525	0.041	0.017	0.017	0.006	0.006	0.006	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

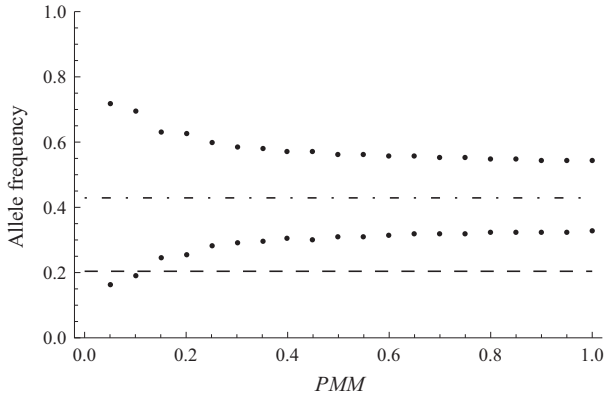

Fig. 4 An example of the results of the stochastic model for one allele, allele 151, in locus HMS3. The dotted dash line represents the allele's frequency in the founding population; the black dots indicate the credible interval (CI) for the expected allele frequency for the most informative test ($\alpha = 0.26$) in each *PMM* value; the dash line indicates the allele's frequency in the current wild population. The allele-frequency shift in this allele did not fall within the CI_α for *PMM* values above 0.15.

re-introduction onset, indicated by substantial shifts in allele frequencies in most alleles. As mating systems could induce increased genetic drift, the drift experienced by the population can provide an opportunity to study its genetic mating system. In this study, we developed a stochastic model simulating genetic drift to study the genetic mating system of the re-introduced wild ass population and to evaluate its potential effect on the preservation of the genetic diversity of the population.

Revealing the genetic mating system using genetic data and stochastic model simulations

The results of the simulation model indicate that, indeed, not all males contributed their genes to the next generation. The most informative results were obtained from the allele-level analysis, indicating a strongly polygynous mating system in which less than 25% of all males participate in the breeding process in each generation, and it is likely that that the actual proportion of mating males per generation is closer to 10%. This strongly polygynous mating system suggests that the population has the potential for strong sexual selection, with a high variance in male reproductive success (see Wade & Shuster 2004 for a formulation of male variance in reproductive success with mating and nonmating males and, additionally, Wade 1979 and Shuster 2009 for the sexual selection consequences of such variance).

The level of reproductive skew in this study is similar to that genetically estimated in other strongly polygy-

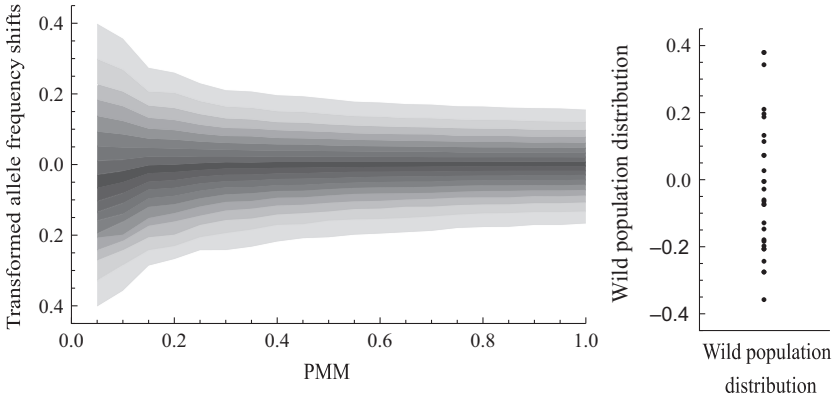

Fig. 5 Distributions of the shifts in the transformed allele frequencies. a) Simulated distributions for different *PMM* scenarios. Each colour shade represents a 5%-quantile of the distribution (lighter shades represent higher shifts in transformed allele frequencies). Lower *PMM* values show more dispersed distributions, indicating stronger genetic drift in these scenarios. b) Distribution of Δa values of the current wild population for 29 alleles. The wild population distribution was compared with the simulated distributions for different *PMM* values (see Fig. 6).

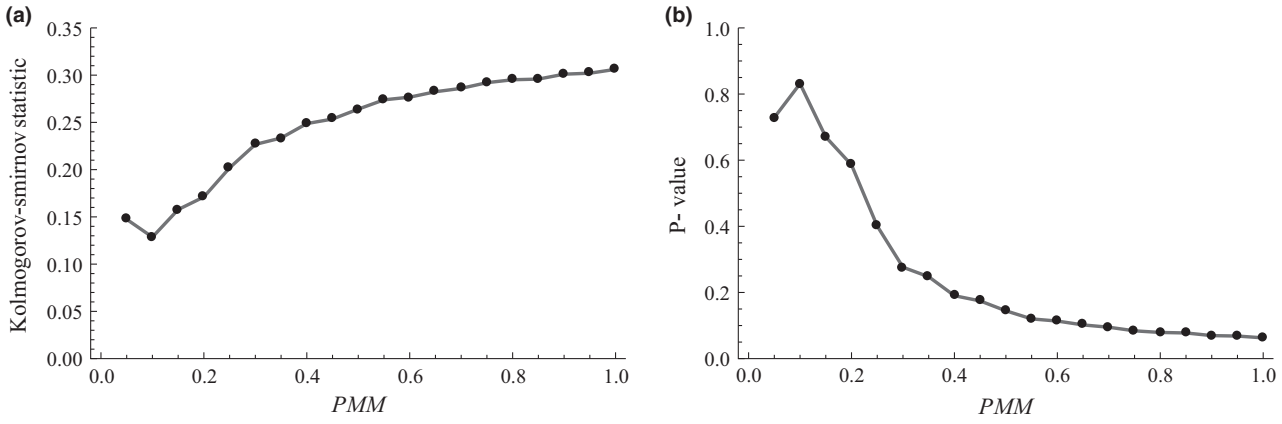

Fig. 6 Goodness-of-fit tests between the distribution of the Δa values of the current wild population and the distributions of Δa values for all *PMM* scenarios (Kolmogorov–Smirnov tests, averaged over 1000 tests). The Kolmogorov–Smirnov test statistic (a) and the corresponding *P*-value (b). Best fit obtained at a *PMM* value of 0.1.

nous mammals, including the Antarctic fur seal (Hoffman *et al.* 2003), the fallow deer (Say *et al.* 2003), the three-toed sloth (Pauli & Peery 2012) and two species of elephant seals (Hoelzel *et al.* 1999). However, these studies, based on paternal analysis, estimated only the 'per year' contribution of males to the gene pool rather than the 'per generation' contribution, estimated here. When measuring the 'per year' contribution of males, in cases where there is a short male tenure, the actual proportion of males contributing to the gene pool could be much greater. The 'per generation' contribution of males to the gene pool, estimated in this study, is rarely calculated in mammals (due to the difficulties in conducting a long-term genetic study that enables a multi-year paternity analysis) although it is more relevant to population genetics and conservation.

The strongly polygynous mating system of the Asiatic wild ass population, as interpreted from the model, is similar and perhaps even more extreme than the mating system assessed from direct observation (27% of the males in the northern Negev Highlands are solitary dominant males, Renan 2014). A stronger genetic mating system than the one observed may indicate that only a portion of the observed dominant males actually mate or that their variance in the number of offspring is greater than that of a Poisson distribution. The mating of 'sneaker' males from bachelor groups has been reported in equids (Bowling & Touchberry 1990; Feh 1999); however, our finding that less than 25% of all males contribute their genes to the gene pool in each generation (which is in concordance with the observed portion of dominant males) suggests that the genetic

contribution of 'sneaker' males in the wild asses is negligible. It is important to note that a fixed *PMM* value was set for each simulation, excluding, in each generation, all other males (1−*PMM*) from mating. In natural populations, although dominant males usually have a higher probability of mating, this probability is not dichotomous and not necessarily fixed over generations. Therefore, the results of our model indicate a mating system that is equivalent to a fixed mating system in which less than 25% of all males mate in each generation. For a detailed discussion on the effect of the model's assumptions on the conclusions, see Appendix S2 (Supporting information).

Measuring genetic drift using shifts in allele frequencies

Two approaches to evaluate genetic drift using allele-level analyses were developed in this study, one based on treating each shift of allele frequency as an independent test and accounting for the independence of within-locus frequency shifts, and the other on a new genetic drift measure using a variance-stabilizing transformation to allow the comparison of all allele-frequency shifts using one statistical test.

When compared to locus-level measures, such as heterozygosity and the number of alleles, the allele-level approaches have several advantages. While H_E is an indirect measure of genetic drift, based on quantifying allele frequencies from a given locus by calculating the probability of two random gametes to be identical, the allele-level analyses quantify the actual shifts in allele frequencies which are the direct effects of genetic drift. In addition, *NA* can only detect genetic drift that is strong enough to induce a loss of alleles, while the allele-level analysis can reveal more fine-scale effects of genetic drift, such as changes in allele frequencies. The failure of *NA* and H_E tests to detect significant differences between the breeding core and wild populations, as well as their inability to provide information regarding the *PMM*, may be a result of their insufficient power to detect the fine-scale effects of genetic drift over a small number of generations. In contrast, a significant change in allele frequencies between the breeding core and wild populations was found using AMOVA, and the allele-level analyses, based on allele-frequency shifts, did yield a significant signal with respect to *PMM*. These results imply that allele-frequency shifts can be far more informative than *NA* or H_E for detecting weak effects of drift.

The allele-level analyses have another major advantage in the context of elusive species. As all other genetic drift measures are per locus measurements, their sample size is usually relatively small. In this study, the sample size of eight loci was not large enough to allow for powerful statistical testing, whereas the statistical power of the allele-level analysis allowed a more detailed and more powerful statistical analysis, and provided significant results. In the study of elusive species, where noninvasive molecular techniques are often used and the number of amplified loci is typically small, this offers an apparent advantage.

Applications of the model to other systems

The stochastic model approach that was developed in the study as an alternative approach to paternity analysis for revealing the mating system of a species can be used in the study of elusive species, where sample collection is limited and noninvasive sampling is often needed (i.e. samples of low DNA quantity and quality). However, it can also be applied to other systems for which researchers encounter difficulty in amplifying a sufficient number of microsatellite loci, or when budget limitations prevent the comprehensive sampling needed for parentage analysis.

For efficient use of the stochastic model approach, at least two time steps of genetic sampling are needed, ideally with a long-time interval between sampling events (enabling enough time for the genetic processes to create a measurable effect). This time interval between sampling is not always easy to achieve; however, this model approach could be applied to several frameworks, such as the study of re-introductions or similar colonization processes and long-term studies. Re-introductions are increasingly becoming a common tool in ecosystem restoration.

As they are usually well-documented and commonly involve long-term study to monitor the re-introduced population, re-introductions have been suggested numerous times as a unique opportunity for 'large scale experiments' that offer exceptional conditions to study evolutionary, genetic and behavioural questions (Sarrazin & Barbault 1996; Seddon *et al.* 2007). As re-introduced populations are often elusive, the simulation model can be used to study these populations, but in doing so, it can also be used as an opportunity to study more general ecological questions. Similar to re-introductions, long-term studies are also becoming common in many fields of ecology (reviewed in Lindenmayer *et al.* 2012). These studies offer an additional well-documented research framework that could benefit from the use of stochastic modelling.

In this study, the simulation approach was used to study the population mating system. However, the model's framework can be further applied to various ecological and population genetic questions. By setting a fixed value of the proportion of mating males, the

model can now simulate the same system under different ecological parameters (e.g. population structure, population growth, etc.), and approximate bayesian computation (ABC) can be used to analyse several parameters simultaneously. Moreover, extending the simulation into future generations can generate specific predictions for the genetic diversity of populations, information that can be used for conservation and management programmes.

Applications for conservation

The genetic diversity levels observed in both breeding core and wild populations (*NA* = 3.6, H_E = 0.56 and *NA* = 3.4, H_E = 0.54, respectively) are lower than the values reported in previous studies on Asiatic wild ass populations in the wild in the Mongolian Gobi Desert (mean *NA* = 9.39, mean H_E = 0.83, Kaczensky *et al.* 2011), as well as in studies on other wild equid populations (plains zebra: *NA* = 6.88, H_E = 0.77, Lorenzen *et al.* 2008; mountain zebra: *NA* = 10.73, H_E = 0.773, Moodley & Harley 2005). However, similarly low levels of genetic diversity were found in 12 captive breeding programmes of Asiatic wild ass (*E. h. onager*) in Europe (mean *NA* = 3.35, mean H_E = 0.83, Nielsen *et al.* 2007). Although caution should be taken when comparing different studies that used different sets of markers, the relatively low genetic diversity of the Negev's population, more similar to breeding core populations than to wild populations, raises concerns.

This low genetic diversity, combined with the strongly polygynous mating system that was detected in the Negev population, is even more worrisome. Although, after four generations since re-introduction, a major reduction in allelic diversity was not observed, substantial shifts in allele frequencies were found. As genetic drift is a gradual process in which shifts in allele frequencies can gradually lead to allele loss, genetic drift induced by the mating system may result in a severe reduction of allelic diversity in future generations. Nevertheless, population subdivision may be developing (Gueta *et al.* 2014; Renan 2014), and future range expansion may accentuate this structuring. This, in turn, may increase the variance effective size (Templeton 2006) and reduce the loss of genetic diversity in the overall population, as has been documented in re-introduced populations of collared lizards (Neuwald & Templeton 2013). In view of the fact that maintaining genetic diversity is crucial for the future viability of the population, a long-term study is needed to monitor the potential effect of the mating system on the genetic diversity of the Negev wild ass population.

In summary, we found that less than 25% of all Asiatic wild ass males participate in the mating process per generation. This is the first time, to the best of our knowledge, that the level of polygyny was measured in wild equids, revealing a strong polygyny level relative to other mammal mating systems documented. We estimated the 'per generation' male contribution to the gene pool, rarely calculated in mammals, which is more ecologically relevant than the 'per year' contribution (typically calculated using paternity analysis) in terms of genetic diversity preservation. The strong effect of the mating system on genetic drift, demonstrated in this study, emphasizes the importance of studying the genetic mating system of small and threatened populations. Our stochastic model approach, accompanied by analyses of allele-frequency shifts, can be applied as an alternative approach to paternity analysis to gain insights into the genetic mating system of these populations.

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S.R. and G.G. equally contributed to the manuscript: S.R. collected and analysed the genetic data. G.G. designed and analysed the stochastic model. S.R. and G.B. developed the study approach and wrote the manuscript; N.S. took a major part in the laboratory work and assisted in the writing; A.R.T. contributed to the statistical analysis; S.B., A.R.T. and A.B. contributed to the research design and conclusions and commented on the manuscript; S.B. conceived the research framework.

Data accessibility

Final genotypes of breeding core population and wild population: uploaded as online supporting information, in Appendix S1 (Supporting information).
All primers used in our study are published ones.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Microsatellite amplification and genotyping protocols.

Appendix S2 Demographic history, model results, sensitivity analysis and assumptions.

Appendix S3 Locus-level analysis – expected heterozygosity and loss of alleles.

Appendix S4 Using the variance stabilizing transformation.