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The impacts of inbreeding, drift and selection on genetic diversity in captive breeding populations

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Abstract

The goal of captive breeding programmes is often to maintain genetic diversity until re-introductions can occur. However, due in part to changes that occur in captive populations, approximately one-third of re-introductions fail. We evaluated genetic changes in captive populations using microsatellites and mtDNA. We analysed six populations of white-footed mice that were propagated for 20 generations using two replicates of three protocols: random mating (RAN), minimizing mean kinship (MK) and selection for docility (DOC). We found that MK resulted in the slowest loss of microsatellite genetic diversity compared to RAN and DOC. However, the loss of mtDNA haplotypes was not consistent among replicate lines. We compared our empirical data to simulated data and found no evidence of selection. Our results suggest that although the effects of drift may not be fully mitigated, MK reduces the loss of alleles due to inbreeding more effectively than random mating or docility selection. Therefore, MK should be preferred for captive breeding. Furthermore, our simulations show that incorporating microsatellite data into the MK framework reduced the magnitude of drift, which may have applications in long-term or extremely genetically depauperate captive populations.

Keywords: adaptation, docility, mean kinship, microsatellite, mitochondrial DNA, pedigree

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Introduction

Captive breeding programmes are often established to maintain population genetic diversity and fitness (Frankham 2008), at least until re-introductions or supplementation of wild populations occur (Utter & Epifanio 2002). Successful re-introductions are exemplified by the black-footed ferret (*Mustela nigripes*), California condor (*Gymnogyps californianus*) and Przewalski's horse (*Equus przewalskii*), but approximately one-third of re-introductions fail (Miller *et al.* 1994; Russell *et al.* 1994; Toone & Wallace 1994; van Dierendonck & Wallis de Vries 1996; Fischer & Lindenmayer 2000). Although poor habitat quality is one common denominator in failed re-introductions (Moorhouse *et al.* 2009), altered behaviours (e.g. courtship rituals, foraging/hunting routine, nest site selection), depleted genetic diversity or a combination of these factors also limit population growth trajectories (Williams & Hoffman 2009).

Adaptation to captivity can be a significant problem for populations that live in artificial environments such as zoos or laboratories (Frankham & Loebel 1992). Although selection for increased fecundity or reproductive success can be useful for short-term demographic gains in captive programmes (Woodworth *et al.* 2002), traits that promote captive reproduction may be maladapted for reproduction in the wild (Lachance & Magnan 1990). Additionally,

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changes in behaviour may have adverse effects. For example, wild antelope sprint when startled, but this behaviour can lead to serious injuries in captive antelope (Lacy 1994). Antelope that survive in captivity may become increasingly sedentary and inured to predators if released. Similarly, aggressive traits that could be beneficial in wild predators may lead to costly agonistic interactions in captivity. The loss of these aggressive traits in captivity could compromise subsequent re-introduction efforts. Whether intentional or unintentional, selection on behavioural traits can result in reduced survival under wild conditions, meaning that minimizing selection in captivity is important to the long-term outcome of captive breeding programmes (particularly if re-introduction is desired; McPhee 2004).

Another significant problem for captive populations is the loss of genetic diversity. Genetic diversity can be lost due to genome-wide processes (i.e. drift and inbreeding) as well as artificial selection on individual genes (i.e. adaptation to captivity; Woodworth *et al.* 2002). The retention of genetic diversity is important to the long-term potential of a population as reduced genetic diversity has been associated with an increased risk of population extinction (Saccheri *et al.* 1998), reduced population growth rate (Hanski & Saccheri 2006) and reduced potential for response to environmental change (Waples 1991). Because these are significant concerns for re-introduced populations, the impact of captive breeding programmes on genetic diversity is of considerable conservation interest.

Genetic diversity is often represented by heterozygosity and allelic diversity. Although inbreeding has no direct effect on allelic diversity, inbreeding directly reduces heterozygosity by increasing the proportion of homozygotes relative to random expectations. In contrast, genetic drift directly affects allelic diversity but only indirectly impacts heterozygosity, because zygosity is a function of the breeding system (Crow & Kimura 1970). These effects are well illustrated with the concept of effective population size (N_e; Allendorf & Luikart 2007), which provides a mathematical framework that can help predict the rate at which genetic diversity is lost. Loss of genetic diversity can be slowed by stabilizing population size (Vila *et al.* 2003), retaining equal number of males and females (Melampy & Howe 1977), maintaining nonoverlapping generations (Crow & Denniston 1988) and equalizing reproductive success (Frankham *et al.* 2006). Nevertheless, populations with small N_e still lose genetic diversity more rapidly than those with large N_e.

Unfortunately, captive populations are often created and sustained to preserve endangered species characterized by small population sizes. Moreover, captive populations are subsequently bottlenecked even further when the population is founded. Thus, most captive populations are small with limited genetic diversity at the outset and are maintained at small sizes due to space constraints in captive facilities. Because these small captive populations are prone to inbreeding and drift, zoo biologists actively seek to maintain N_e as large as possible within resource constraints (Lacy 2012). Often, this is accomplished by utilizing breeders that are likely to harbour underrepresented alleles by selecting relatively unique individuals (i.e. individuals with the lowest mean kinship; Fernandez *et al.* 2004). Mean kinship is the probability that an allele sampled from an individual is identical by descent with an allele, at that particular locus, that is sampled at random from the population (Ballou *et al.* 1995). In other words, a mean kinship value is a numerical representation of the genomic uniqueness of an individual in a population, based on ancestry. Theory predicts that selecting breeders that minimize each generation's mean kinship should reduce the rate of population evolution by more effectively retaining genetic diversity compared to random mating (Frankham 2008; Ortega-Villaizan *et al.* 2011; Ivy & Lacy 2012). However, this theory remains largely untested empirically (Williams & Hoffman 2009).

We used replicate experimental populations of white-footed mice (*Peromyscus leucopus*; Rafinesque 1818) to compare the genetic diversity patterns between three breeding protocols—random mating (RAN), minimizing mean kinship (MK) and docility selection (DOC). We compared the change in heterozygosity and allelic diversity in these experimental populations to each other, to the natural source population and to theoretical expectations derived from computer simulations. We did so using microsatellite and mtDNA loci as well as actual pedigrees; we simulated the expected loss of diversity by modelling the breeding protocols and simulating the effect of combining pedigree and microsatellite data in breeding pair selection. Based on previous theoretical simulations of various breeding protocols (Ballou *et al.* 1995; Sonesson & Meuwissen 2000), we predicted that the MK lines would have reduced inbreeding relative to the RAN and DOC populations and that, because the selection imposed on the DOC populations may be more likely to select related individuals that exhibit a particular phenotype, the DOC populations would have the greatest accumulation of inbreeding. Specifically, we predicted the retention of genetic diversity in MK > RAN > DOC within each generation. Finally, we used simulations to assess the effectiveness of combing genetic marker and pedigree data in conserving genetic diversity, relative to pedigree-based breeding protocols.

Materials and methods

The complete history of our captive populations is available in Lacy *et al.* (2013). Briefly, captive colonies were founded with 20 white-footed mice trapped in Illinois at Volo Bog State Natural Area in 2001. As in most captive breeding programmes, we assumed for all pedigree calculations that the wild-caught mice were neither related nor inbred. Offspring (usually 24) from each of the 10 founding pairs were divided equally into six breeding groups that were maintained for 20 generations. Beginning with the first generation of captive born individuals, 20 pairs of mice from each population were chosen for mating following the RAN, MK or DOC breeding protocols. Under the RAN protocol, mice were selected for breeding without regard to phenotype or genotype, although inbreeding of close relatives (more closely related than the average pairwise kinship in the population at a given time) was avoided. The MK protocol selected for breeding those individuals with the lowest, pedigree-calculated MK values to (in theory) maximize the retained genetic diversity. This was carried out by calculating pairwise kinship values, obtaining a MK for each individual by averaging its kinship to all living animals (including itself) in its population, removing individuals with the least desirable (highest MK) values, recalculating and repeating until all individuals were removed and then assigning pairs from the last 20 males and 20 females remaining. These would be the subset with the lowest overall MK, as estimated by the ranked MK procedure (Ivy & Lacy 2012). Finally, the DOC protocol quantified presumed stereotypic behaviours (as measured by time spent gnawing at cage bars and flipping at night) and used these values as an indicator of docility. In other words, more sedentary individuals were assumed to be more docile relative to the other individuals in the replicate population. The lowest scoring male was paired with the lowest scoring female, second-lowest male with second-lowest female, etc. Thus, the most docile animals were mated to imitate the selection for docility that can occur in captive breeding programmes. The MK and DOC protocols avoided close inbreeding in the same manner as the RAN protocol. In total, six populations were maintained (two replicates for each protocol), and animals maintained in different lines were never interbred through the generations analysed in this study. Pedigrees were maintained in an Access (Microsoft Corp.) database program developed by one of us (RCL) for the maintenance of records on research animal colonies, and genetic calculations on the pedigrees were performed with the PMX software (Lacy *et al.* 2012). The Institutional Animal Care and Use Committee of the Chicago Zoological Society approved all trapping, housing and husbandry protocols.

Pedigree analysis

We analysed the pedigrees to estimate the inbreeding coefficient (F) and gene diversity (GD, the heterozygosity expected under Hardy–Weinberg equilibrium) for each population at each generation. We calculated F using the calcInbreeding function in the R (R Development Core Team, http://www.R-project. org) package pedigree (Coster 2013) and used the function kinship in the package kinship2 (Therneau *et al.* 2014) to calculate the *mk* for each individual, averaged over each population/generation combination. For F and the population average *mk*, we used the standard error to determine the 95% CI and compared these values across generations and between populations. Additionally, we used the equation

$$GD = 1 - \overline{mk} \qquad \text{(eqn 1)}$$

to compute gene diversity (relative to the source population) using the upper and lower values of 95% CI of population average mean kinship values at each generation (Lacy 1995). Finally, we calculated the relative GD present at each generation by dividing the GD range estimates by the initial GD present in the original 10 founding pairs.

Microsatellite and mtDNA analysis

Molecular assessments of diversity and kinship require data from many independent loci, and because we maintained six captive populations for 20 generations, there were far too many mice to exhaustively genotype. Thus, we sampled representative time points to establish trends associated with genetic diversity by taking a small clip of tail tissue from carcasses or by ear punch tissue taken during trapping. From the captive populations, we sampled all 20 original founders. We also sampled 17–23 individuals from each of the six captive populations at the 6th generation, and 22–25 individuals from the five extant captive populations (one DOC replicate failed to reproduce after generation 9) at the 19th generation. Finally, we sampled 25 individuals from the wild, source population, which were collected at the end of the captive breeding programme (2012).

We extracted DNA following a standard phenol–chloroform procedure (Sambrook & Russell 2001) and amplified each sample at 11 microsatellite loci (Schmidt 1999; Chirhart *et al.* 2000; Prince *et al.* 2002; Mullen *et al.* 2006) using polymerase chain reaction (PCR) on an Eppendorf Mastercycler (Eppendorf Westbury, New York). We performed multiplex PCRs in 20 μL reactions, using annealing

temperatures of 54 °C or 51 °C (BW4-129, PLGT67, PML04, PML06, PML10, PML12, PO-35, PO3-68, PO3-85 at 54 °C; BW4-178, PLGT15 at 51 °C) and 40 ng template DNA, NEB Taq polymerase (1U), 1.5 mM MgCl2, 10 mM Tris-HCl, 50 mM KCl, 0.5 mg/mL BSA and 0.2 mM of each dNTP. Finally, we used an ABI 3130XL and GeneScan4.1 (Applied Biosystems, Foster City, CA) to determine genotypes of each individual at each locus.

We used the microsatellite data to critically test for different evolutionary trajectories in each population. We computed observed microsatellite heterozygosity and obtained the bootstrapped (n = 1000) 95% CI for each population and generation (bootstrapHet function, PopGenKit; Paquette 2012). We also calculated allelic richness across each genotyped generation and population with a bootstrapped 95% CI (Davison & Hinkley 1997; Canty & Ripley 2014) across individuals in each sampled generation. Finally, we calculated the relative allelic richness at each generation by dividing the minimum and maximum allelic richness values from the 95% CI estimates at each generation by the average number of alleles observed in the initial 10 founding pairs.

Additionally, we used a 382-bp mitochondrial DNA (mtDNA) d-loop region (MD1 and 12ST; Morzunov *et al.* 1998) to estimate matriline diversity. We employed 20 μL PCR reactions using 30-ng template DNA, 1U NEB *Taq*, 0.5 μM the forward and reverse primer, 2.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 0.5 mg/mL BSA and 0.2 mM of each dNTP. We amplified the locus using the following cycling conditions: 94 °C for 30 s; 30 cycles of 94 °C for 30 s, for 59 °C 1 min, and 72 °C for 1 min; and a final 5 min extension at 72 °C. We performed dideoxy sequencing reactions using BigDye, an ABI 3730XL sequencer (Applied Biosystems, FosterCity, CA, USA), and Sequencher 5.0 (Gene Codes Corp., Ann Arbor, MI, USA) to align and edit sequences. Representative offspring from each of the original founding pairs of mice were sequenced and haplotypes assigned to each individual in a matriline as determined from the pedigree. We characterized individuals from the wild, source population as well as each captive population from generations 1–19 and then determined haplotype richness by counting the number of haplotypes present at each time period. We also determined the maximum number of haplotypes present, assuming each founding female possessed a unique haplotype, by counting the number of maternal lineages present at each generation. Haplotype diversity at each generation was calculated using the equation

$$H = 1 - \sum_{i=1}^{j} p_i^2 \qquad \text{(eqn 2)}$$

where p is the frequency of the ith haplotype (Nei & Tajima 1981). Finally, we quantified the nucleotide diversity (π) present in the wild and captive populations using the equation

$$\pi = 2 * \sum_{i=1}^{n} \sum_{j=1}^{i-1} x_i x_j \pi_{ij} \qquad \text{(eqn 3)}$$

where n is the number of haplotypes, x_i and x_j are the frequencies of the ith and jth haplotypes, respectively, and π_{ij} is the number of nucleotide differences between the ith and jth haplotypes (Nei & Li 1979). We compared haplotype richness, haplotype diversity and nucleotide diversity measures across generations by comparing the 95% CIs calculated using 1000 bootstraps across individuals.

Simulation analysis

We simulated the loss of neutral genetic diversity in the captive populations to provide a theoretical baseline for comparison against the observed loss of diversity. The R simulations began with the empirical multilocus genotypes and coded mtDNA haplotypes of the 20 wild mice used to found the captive populations. We determined the number of offspring produced using data collected from the captive populations, specific to each breeding protocol, by randomly drawing the number of offspring produced by each simulated parent pair from the observed distribution of offspring successfully weaned by parent pairs in the captive populations. This simulation procedure replicated the mean and variance in reproductive success by pairs that occurred in the captive populations. To generate offspring genotypes, we randomly selected one allele at each locus from each parent, and to generate offspring mtDNA haplotypes, we assigned the haplotype of the simulated mother. We assigned male/female with a probability of 0.5 for either sex.

We selected breeders following the captive population breeding protocols. We simulated the RAN populations by randomly pulling individuals from the list of all possible offspring without replacement. However, just as in the captive study, potential pairs with a higher kinship than the average for the entire breeding pool were rejected. To simulate the MK populations, we chose breeding pairs following the MK protocol utilized in the captive study (kinship2 package; Therneau *et al.* 2014). Finally, to simulate DOC populations, we used a simple additive model with one locus and two alleles for flipping and a second (unlinked) biallelic locus for gnawing. We randomly assigned, with equal probability, alleles to each wild-caught parent for both docility loci. We scored behaviour by drawing from a normal distribution with a genotype-specific mean. For flipping and gnawing, scores for individuals homozygous for

one allele were drawn from a distribution with a mean of zero, heterozygotes a mean of 0.4, and homozygotes for the other allele from a mean of 0.8. This scheme allowed some variance in the behaviour scores to account for variability within the observed behaviour scores and, on average, scored individuals with more copies of the high-scoring allele higher than those with fewer or no copies. Finally, we summed the behaviour scores and, to select docile individuals, chose individuals with the lowest combined scores, and paired individuals as in the captive populations.

For all scenarios, we simulated 20 generations beyond the initial founding. We incorporated microsatellite mutation into the models by allowing stepwise mutations at a rate of $\mu = 10^{-3}$ per generation (Dallas 1992; Bulut *et al.* 2009) during allele selection for all simulated individuals. We simulated 100 replicate populations using a random draw (with replacement) from the distribution of offspring produced under each protocol for each of the simulated populations. For each generation, we calculated the inbreeding coefficient from the simulated pedigree (kinship2 package; Therneau *et al.* 2014), allelic richness across the simulated genotypes for all 11 loci, haplotype diversity using equation 1 and the number of haplotypes remaining in the population at each generation.

We compared the 95% CIs of the simulated results to the 95% CIs of the empirical data, calculated using the replicate lines for each protocol, to look for evidence of associative overdominance and selective sweeps on functional genes linked to our markers. We expect that if the mode of selection was a selective sweep, the loss of alleles should occur more quickly in the captive populations than in the (neutral) simulated data due to hitchhiking. However, we expect that if the mode of selection is associative overdominance, the rate of allele loss in the empirical data will be slower than the simulated data (Montgomery *et al.* 2010).

Enhanced MK protocol

The effect on long-term breeding programmes of assuming unrelated founders is thought to be small (Rudnick *et al.* 2009), but the effect of sibling differentiation is unknown. We utilized simulations to investigate the impact of including microsatellite genotypes within the MK breeding protocol. mean kinship values, as calculated from a pedigree, assign equal uniqueness to many individuals early in a pedigree and to all offspring of the same breeding pair, even though early individuals may not be equally related (Ballou *et al.* 1995) and sibling genomes differ (on average) at 50% of loci (Fisher 1919). Our microsatellite genotypes undoubtedly do not reflect the diversity of each genome with complete accuracy (DeWoody & DeWoody 2005), but utilizing the microsatellite data contained in individual genotypes may be useful as a model for improving the MK protocol with genome-wide genotypic data.

We simulated the effect of incorporating microsatellite genotypes as a measure of uniqueness among siblings or other individuals with the same MK score. Again, we ran our simulations in R, but this time incorporated a two-step procedure to select breeders. First, as in the MK protocol outlined above, we calculated mean kinship and determined the highest mean kinship individual or individuals. If the lowest mean kinship score was shared between multiple individuals, we implemented a second step of breeder selection where we identified and eliminated the individual with the lowest microsatellite uniqueness (among individuals identified in the first phase of breeder selection) calculated from the simulated microsatellite genotypes. By continuously eliminating individuals that scored the poorest in terms of kinship and microsatellite relatedness, we screened the population for the most unique individuals in terms of both pedigree and marker data. As we did in the MK protocol, this two-step process was repeated until all individuals were removed from the breeding pool, and the 10 males and 10 females that were removed last were selected as simulated breeders for the next generation. We modified a portion of the relatedness estimator of Queller & Goodnight (1989) to calculate an estimate of genetic uniqueness by targeting individuals with rare alleles, relative to the individuals in the extant population. We estimated the genetic uniqueness score for each individual (s_i) using the equation

$$s_i = \sum_{l=1}^{r} \sum_{a=1}^{t} (0.5 * n_k - p_k) \qquad \text{(eqn 4)}$$

by summing across loci (l) from 1 to r, the sum of the frequency (p) of the kth allele, across the alleles (a) from 1 to t at each locus within each individual, weighted by the number of copies of allele k (n_k) within the individual. As in the previous MK simulations, the 10 males and 10 females eliminated last were used as simulated breeders for the next generation. We simulated 100 replicate populations and calculated the allelic richness and compared the enhanced protocol to the MK protocol using the 95% CI at each generation.

Results

All captive populations were successfully bred according to the assigned protocols. Although the second DOC population expired due to reproductive failure after generation 9, all other populations were maintained through generation 20.

Pedigree analysis

Pedigree estimates of average inbreeding indicated an increase in inbreeding over the course of the study (Fig. 1A). A rapid increase occurred in one of the DOC lines between generations 5 and 9, and that population collapsed due to reproductive failure. The loss of the second DOC population suggests that fitness was reduced by inbreeding depression [see Lacy *et al.* (2013) for details of the reproductive trends]. The remaining DOC population and both RAN populations accrued inbreeding at the same rate throughout the duration of the study. Although inbreeding increased in all populations, the increase was significantly slower in the MK populations; the inbreeding coefficients in the MK lines were significantly different from each other at generations 11–19 (MK1 > MK2) and significantly smaller than all other lines from generations 16–19 (Fig. 1A).

Concomitant with increased inbreeding, GD decreased in all populations. Although the pattern of loss of GD mirrored the pattern of increase in inbreeding—the smallest loss of GD occurred in the MK lines, while the largest loss occurred in the DOC lines—we observed slight differences in the timing of significant differences. We found significantly less GD in the second DOC line at generations 7–9 and significantly higher retention of GD in the first MK population beginning at generation 9 (Fig. 1B).

Microsatellite and mtDNA analysis

Genetic diversity, as measured by microsatellites, declined in each captive population relative to the wild source population. Observed heterozygosity decreased by an average of 10% (max 13%) by generation 19. Furthermore, the magnitude of the decrease in heterozygosity between all of the captive populations and the wild source populations was small (Fig. 2A). There is also evidence that the MK strategy successfully reversed the effects of unequal breeding success in early pairings, as heterozygosity in the MK lines was not significantly different between generations 6 and 19, but the relative value increased.

In addition to decreases in heterozygosity, all populations lost alleles relative to the original founders and to the contemporary wild source population. The MK treatment resulted in retention of more alleles than in the captive RAN and DOC treatments and, in general, the RAN populations retained more alleles per generation than the DOC lines. However, the bootstrapped 95% CIs overlapped between protocols within a generation (Fig. 2B). Additionally, the captive populations did not retain a large proportion of the allelic diversity found in the source population. The difference in the relative rate of loss of microsatellite genetic diversity compared to the pedigree-calculated GD suggests that the panel of microsatellites we utilized did not accurately convey the full diversity of the genome (Fig. 2; DeWoody & DeWoody 2005).

Regarding the mitochondrial DNA results, the 25 individuals sampled from the wild population contained seven haplotypes defined by eight variable sites whereas, among the 10 founding females, we identified four haplotypes defined by four variable sites (GenBank Accession nos. KP137579-KP137587). Between the wild and captive populations, two haplotypes were shared. Two of the captive populations (RAN1 and MK2) maintained haplotype and nucleotide diversity until the last generation, but all other lines ended the study with only one haplotype remaining (Fig. 3). The maximum possible number of haplotypes present, as measured via the maternal line, matched the overall pattern of loss of

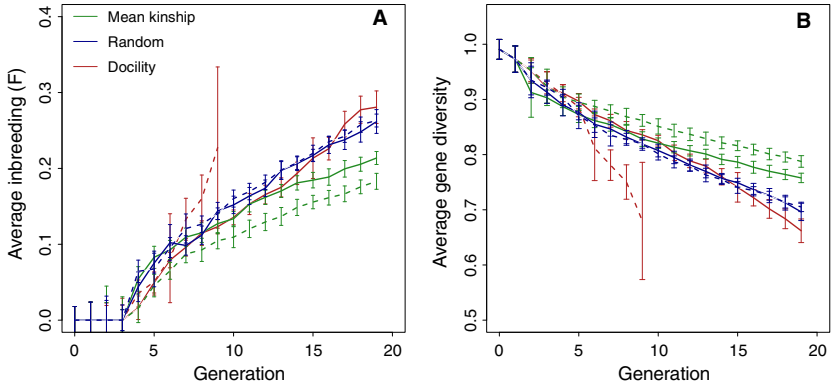

Fig. 1 Inbreeding (A) and gene diversity (GD) (B) over 19 generations as calculated from the pedigree. For all lines, inbreeding increased and GD decreased in all generations, although the largest change was observed in the lines bred using the docility protocol. GD is shown relative to the GD observed in the founding pairs. Solid lines indicate replicate one and dotted lines indicate replicate two for each breeding protocol. Error bars indicate 95% CI across individuals in each population and at each generation.

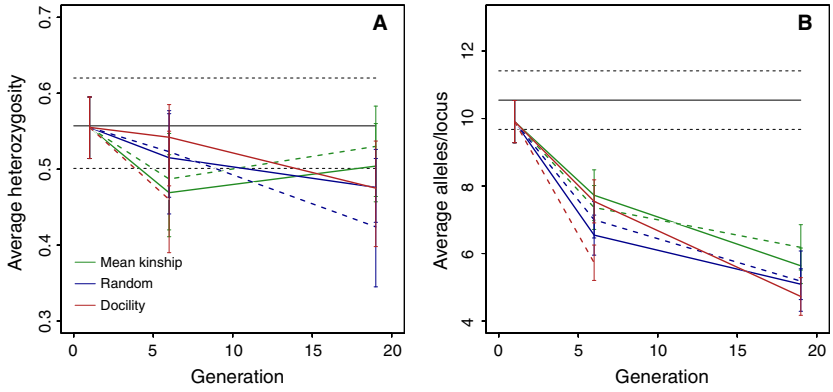

Fig. 2 Microsatellite heterozygosity (A) and allelic richness (B) measured in the empirical data set. Overall, heterozygosity declined very little and the number of alleles declined significantly relative to the wild population for all protocols. Solid coloured lines indicate replicate one and dotted coloured lines represent replicate two for each breeding protocol. Error bars indicate the bootstrapped 95% CI. The wild population is displayed in black, horizontal lines where the mean is shown with a solid line and the extent of the bootstrapped 95% CI is indicated with dotted lines.

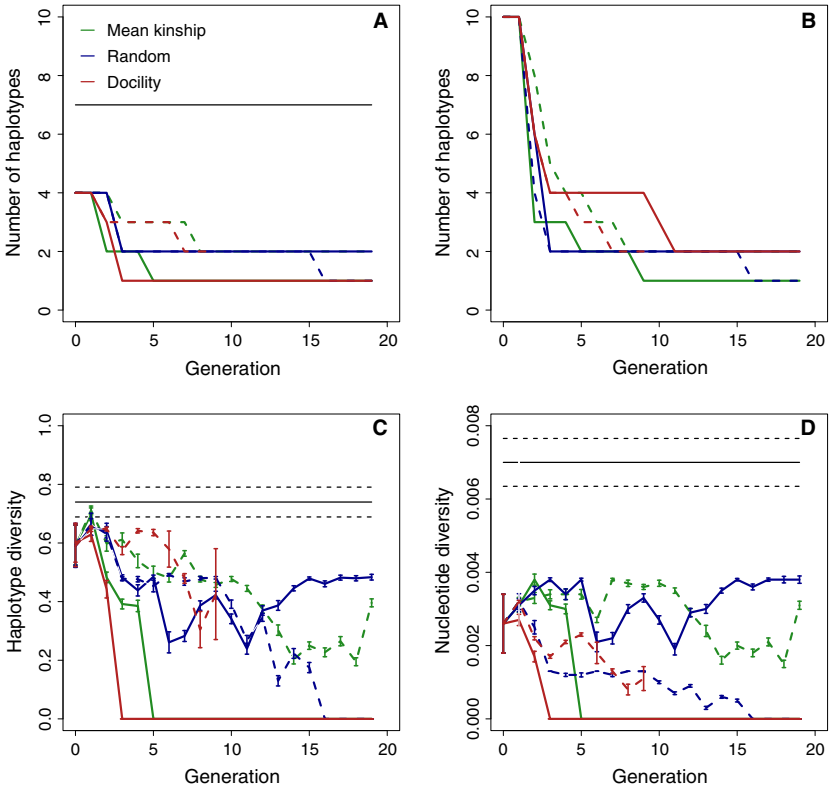

Fig. 3 Mitochondrial diversity as measured via a 382-bp section of the d-loop. Number of haplotypes (A) declined and ended with a maximum of two haplotypes in each population. This is contrasted with the maximum number of haplotypes possible, if all founding females possessed unique haplotypes (B). Haplotype diversity (C) and nucleotide diversity (D) are shown relative to the wild source population. In all panels, solid coloured lines indicate replicate one and dotted coloured lines represent replicate two for each breeding protocol. Error bars indicate the bootstrapped 95% CI. The wild population is displayed as the horizontal black lines, where the mean is shown with a solid line and the extent of the bootstrapped 95% CI is indicated with dotted lines.

d-loop haplotypes. Additionally, all captive populations had significantly lower diversity relative to the wild population by generation three and, by generation eight, all of the captive populations were significantly different than the captive founders in terms of haplotype diversity (Fig. 3). Because the haplotypes varied significantly among replicate lines, breeding protocol did not appear to impact the retention or rate of loss of mtDNA haplotype richness and diversity.

Simulation analysis

Our simulations of the captive populations matched the general trends observed from the pedigree: kinship increased in all populations, with the smallest increase in MK populations (Fig. 4). Our simulations of the DOC behaviour selection protocol did not completely capture the range of loss of diversity we observed in the empirical data, perhaps because the simple model we utilized

to represent genetic control of the behaviours under selection was not sufficiently complex (i.e. too few loci and alleles). That said, our simulations generally supported the trends observed in the empirical data. Populations simulated using the MK protocol retained more alleles than populations simulated under the RAN or DOC protocols (Fig. 5). Additionally, we observed significant differences between the MK and RAN populations in terms of haplotype richness and diversity, at least for a small number of generations (Fig. 6). However, by the 19th generation, there was no difference in mtDNA diversity remaining.

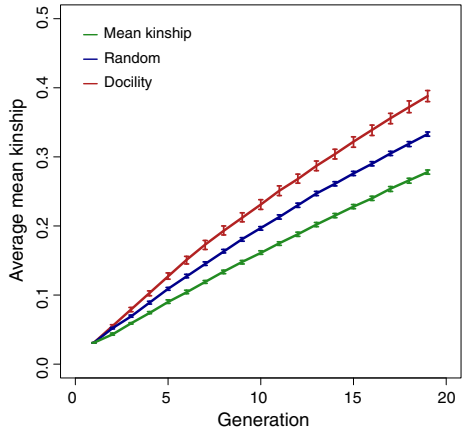

Fig. 4 Average mean kinship from simulated populations utilizing the three breeding selection protocols. After generation two, all protocols had significantly different average mean kinship. Error bars show 95% CIs for 100 replicate populations for each protocol.

Enhanced MK protocol

We investigated the impact of adding microsatellite genotype data to the MK breeding protocol via simulations. We found that, by generation 8, the microsatellite-enhanced MK protocol retained significantly more alleles than the traditional MK protocol (Fig. 7A). Presumably, this is due to the weight the second-step breeder selection protocol (equation 4) placed on individuals with more rare alleles, relative to individuals with equal MK values. However, this increase came at a cost of slightly increased inbreeding in the enhanced MK populations relative to the MK populations (Fig. 7B).

Discussion

We employed a multi-faceted approach that included analysis of the pedigrees, microsatellite genotyping, mtDNA sequencing and simulation of captive populations to compare three different breeding protocols that

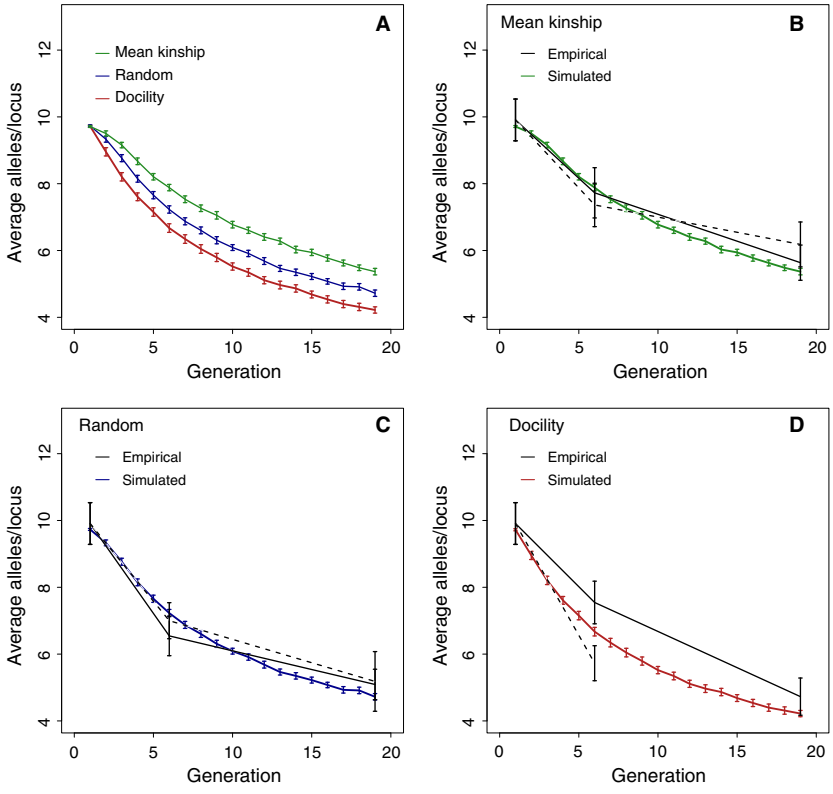

Fig. 5 Simulated allelic richness (A) and empirically estimated allelic richness from 11 microsatellite loci compared to simulated allelic richness for the mean kinship (B), random mating (C) and docility selection (D) protocols. In all panels, solid coloured lines indicate replicate one and dotted coloured lines represent replicate two for each breeding protocol. Error bars indicate the bootstrapped 95% CI in the empirical data and the 95% CI in the simulated data. While the simulated number of alleles was significantly different between breeding protocols after generation two, the only significant difference between the simulated data and the empirical data was observed in the docility-selected population at generation six.

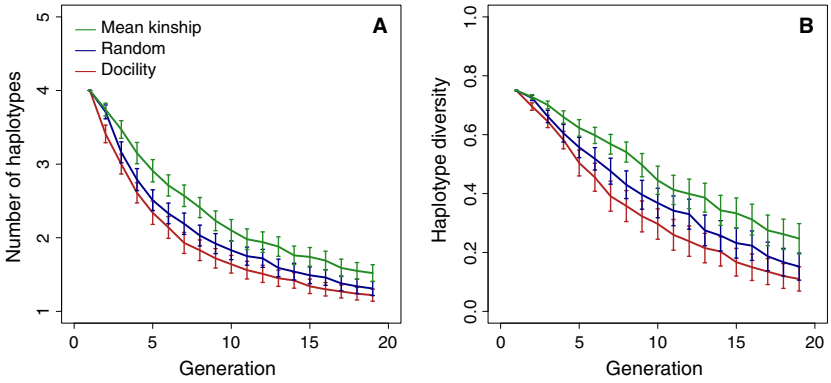

Fig. 6 Number of haplotypes (A) and haplotype diversity (B) from simulated populations utilizing the three breeding selection protocols. Error bars show 95% CIs for 100 replicate populations for each protocol. Although the number of haplotypes and haplotype diversity was typically significantly different between the mean kinship and docility protocols from generations 5–20, the random population 95% CIs generally overlapped the 95%CIs for the MK and docility populations.

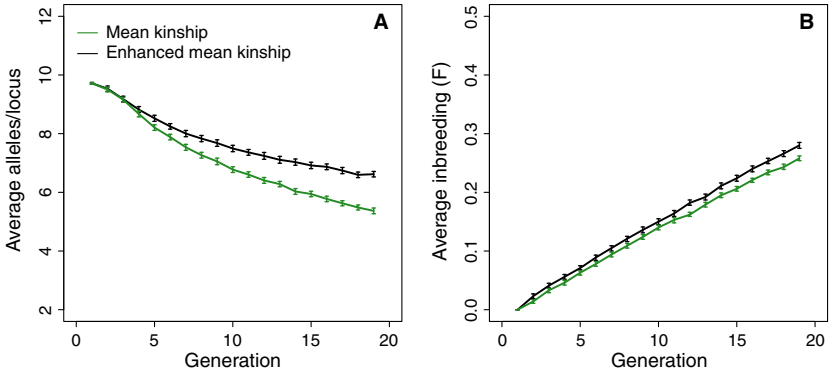

Fig. 7 Comparison of (A) the average alleles per locus and (B) average inbreeding in the populations simulated using the mean kinship (MK) protocol and the MK protocol enhanced with microsatellite data. Error bars show 95% CIs. Utilization of microsatellite data within the MK breeder selection protocol resulted in the retention of additional alleles, although inbreeding also increased.

might be used to propagate wildlife with the goal of retaining genetic variation. In all populations, we observed an increase in the inbreeding coefficient and a loss in microsatellite diversity over time (Fig. 1). We observed that minimizing MK consistently resulted in the retention of the most genetic diversity, based on data from the pedigree (Fig. 1), microsatellites (Fig. 2) and our simulations (Fig. 4). The matrilineal mtDNA haplotype diversity and richness retained was similar across breeding protocols (Fig. 3), possibly due to the decrease in effective population size, as mtDNA effective population size is one-quarter of that of nuclear DNA. We found that the RAN populations lost genetic diversity more quickly than the MK populations and more slowly than the DOC populations that were subjected to artificial selection for behavioural traits (Figs 1 and 2). Finally, we noted that the DOC protocol resulted in the largest divergence among replicates in genetic diversity retained (Figs 1, 2 and 3). Our results reinforce the use of a breeding protocol that minimizes MK, as the reduction in loss of GD under the protocol to minimize MK results in a nearly doubled effective population size (Ballou *et al.* 1995).

Our estimate of genome-wide changes, as calculated from the pedigree, suggested significantly different evolutionary trajectories between the breeding protocols. These results are in agreement with theoretical MK estimates (Ballou *et al.* 1995) as well as the other documented example of empirical data comparing MK and RAN populations over several generations, which utilized *Drosophila* populations (Montgomery *et al.* 1997). However, the magnitude of the difference in the inbreeding coefficient between the RAN and MK lines in the final generation of the *Drosophila* population (~0.5 and ~0.3, respectively) was greater than in our *Peromyscus* (~0.3 and ~0.2, respectively). Although there were differences between the populations other than species utilized—for example, higher inbreeding levels at the outset of the breeding protocols, fewer breeding individuals at each generation—the difference in the accumulation of inbreeding may be indicative of pairs failing to mate, particularly early in the captive programme. In other words, reproductive failures among the early pairs of mice lead to a reduction in founder genomes that could not be recovered, and this leads to less effectiveness of the MK protocol to retain diversity

and avoid accumulated inbreeding than may have otherwise been expected based on the *Drosophila* model. This is also evident in the plot of maximum possible haplotypes at each generation (Fig. 3B), which shows the number of haplotype linages present at each generation. In the first few generations, many lineages were lost due to failure of a pair to reproduce or the chance event excluding females in the small number of pairings.

For all populations, we observed an overall slow decline in microsatellite heterozygosity values across generations (Fig. 2). The proportion of heterozygosity included in a captive population of founders can be predicted by the equation

$$H_f = H_w\left(1 - \frac{1}{2N}\right) \qquad \text{(eqn 5)}$$

where H_w is the heterozygosity of the source population, N is the number of founders, and H_f is the heterozygosity of the founders (Crow & Kimura 1970). Using an $N = 20$, our initial founding individuals were expected to capture 97.5% of the heterozygosity present in the wild populations (Ivy & Lacy 2010), which is similar to what we observed between the wild population and the original founders (Fig. 2). This suggests that using 10 pairs was sufficient for initially capturing much of the existing GD present in the wild population. However, heterozygosity is expected to decline in any closed population (Allendorf & Luikart 2007). Using the equation

$$H_t = H_0\left(1 - \frac{1}{2N}\right)^t \qquad \text{(eqn 6)}$$

where t is the number of generations and H_t is the heterozygosity of the population at time t (Crow & Kimura 1970; Fisch *et al.* 2013), the proportion of heterozygosity remaining in the population after 20 generations is expected to be ~78% of the heterozygosity present in the original 10 founding pairs if the effective population size was equal to the number of breeders ($N = 40$). According to the microsatellite data, the randomly mating captive populations retained ~80% of the starting heterozygosity over the course of 20 generations (Fig. 2). Thus, the observed loss of heterozygosity was very close to the theoretical expectation, suggesting that variance in family sizes was approximately Poisson. Additionally, retention of heterozygosity immediately after a bottleneck may be expected as the increase in frequency of a rare allele (relative to the wild population) leads to an associated increase in heterozygosity (Leberg 1992).

In contrast to heterozygosity, we observed a rapid decrease in microsatellite average alleles per locus. As predicted by theory and simulations (Ballou *et al.* 1995; Sonesson & Meuwissen 2000), the populations with the fewest alleles lost were the MK lines followed by RAN and finally DOC populations, although the differences between the populations were not significant. Thus, the loss of alleles suggests that all of the captive breeding protocols tested do not effectively retain alleles. Typically, the loss of alleles is slowed by maintaining a large captive population (Forstmeier *et al.* 2007). However, captive populations in general and captive populations of endangered species in particular are typically small owing to the practicalities associated with limited space, resources and often individuals. Therefore, retention of the maximum possible amount of genetic variation captured in the founding population is required as mutations do not accumulate at a rate sufficient to account for the loss of alleles due to drift, at least at neutral loci.

We used a theoretical backdrop to account for the effects of mutation, migration and genetic drift by comparing the observed microsatellite diversity in the *Peromyscus* populations to simulated microsatellite diversity. We added the effects of mutation by allowing stepwise mutations to occur during the simulated meiosis step and drift by replicating the population size and selection of breeders for each generation. Because the different captive populations never interbred, migration had no effect on the theoretical or empirical data. Therefore, the observed deviation in diversity between the theoretical and empirical data should be due primarily to selection.

Our empirical and simulated results were similar, although there were differences in the average number of alleles per locus present at generation six in the docility populations (Fig. 5). Differences in the docility population may simply be due to the considerable additional stochasticity that occurred as the population was in collapse, whereas differences between the simulated and empirical data in the random and MK lines, had they been observed, may have represented unintentional selection that occurred within the randomly mated lines. Because adaptation to captivity may mean that individuals are maladapted to survival and reproduction in the wild (Woodworth *et al.* 2002; Williams & Hoffman 2009), reducing the accumulation of selection and subsequent effects is important in captive populations (Leus *et al.* 2011).

Relative to the genome-wide genetic diversity estimates obtained via the pedigree, our microsatellite and mitochondrial data showed a more rapid loss of genetic diversity in the captive populations (Figs 1, 2 and 3). One possible explanation for this discrepancy is that a selective sweep on genes near the genotyped microsatellites increased the rate of loss of diversity. Although this has been observed in other captive populations where genetic markers near genes were targeted (Montgomery *et al.* 2010), our simulated results of the

microsatellite data are correlated with the empirical data (Fig. 5), suggesting that selection was not the cause of the difference in the pedigree and microsatellite trends. It is also possible that populations lose neutral diversity faster than the loss predicted by the pedigree. While we cannot rule out pedigree imprecision, the successful use of pedigrees in maintaining stable populations seems contradictory to widespread errors resulting from the use of pedigrees (Stanley Price 1989; Russell *et al.* 1994). Instead, we posit that in small captive or wild populations, cumulative random drift across generations leads to considerable deviations from theory in allele retentions, allele frequencies and heterozygosity at small numbers of loci that very incompletely sample the genome. This suggests that the rate of loss of allelic diversity is more unpredictably affected by drift than the rate of loss of heterozygosity is impacted by inbreeding.

Particularly with respect to minimizing MK breeder selection protocols, pedigree-based methods have proven effective at minimizing the loss of genetic diversity (Lacy 2009) although inbreeding depression still occurs in some captive populations (Boakes *et al.* 2007). We utilized simulated microsatellite genotypes to increase the effectiveness of the MK breeding protocol. While our protocol resulted in an increase in the retention of alleles at the simulated loci, the additional selection step also resulted in a small but significant increase in inbreeding (Fig. 7). Our results mirror those obtained using a similar approach, in which a related MK protocol was modified using Ritland's relatedness estimator (Ritland 1996), although a direct comparison of inbreeding (as measured by the pedigree statistic F) between the MK and the microsatellite-modified protocol was not conducted (Doyle *et al.* 2001). In any population, selecting for rare alleles at any set of 11 loci is unlikely to result in an increase in rare alleles across the genome, and selecting on very few loci may lead to the depletion of variability at other loci (Lacy 2000). However, as our estimates of genome-wide diversity become more precise, distinguishing between individuals using genome-wide markers should result in genome-wide retention of alleles. While such extensive genotyping may not be worth the effort and cost associated with these tasks in many situations, the use of genomic markers and a pedigree in particularly genetically depauperate populations may lead to greater success in captive breeding and re-introduction efforts.

Conclusions

The conservation of threatened and endangered species may require the establishment of captive breeding programmes. Our research indicates that the minimized MK breeding protocol retains genetic diversity more effectively than random mating (RAN) or docility selection (DOC), suggesting that the MK protocol should be the preferred method in maintaining GD in captive populations. However, the MK protocol does not fully mitigate the effect of drift, as illustrated by the loss of about half of the microsatellite alleles over 19 generations. Using simulations, we have shown how microsatellite data can be used to help differentiate between individuals with the same kinship estimate and thus reduce the impact of drift. These findings have the potential to slow the loss of genetic diversity in captive populations, reduce inbreeding depression and effectively increase the fitness of threatened and endangered species maintained in captivity. This, in turn, should improve the success of these populations once repatriated into the wild.

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J.R.W. and J.A.D. are interested in the use of genetic markers to address population and conservation issues; J.A.I. and R.C.L. routinely design and implement captive breeding strategies for endangered species. J.R.W., J.A.I., R.C.L. and J.A.D. designed the molecular assays and simulations whereas J.A.I. and R.C.L. conceived and bred the original captive populations. J.R.W., N.B.F., M.C.L. and R.C.L. collected the empirical data. All authors were involved in the analysis and writing the manuscript.

Data accessibility

Sequence data for this study available via GenBank (Accession nos. KP137579–KP137587) and pedigree, microsatellite genotypes, mtDNA sequences, as well as simulation R code, input files and example output files are available via Data Dryad (http://dx.doi.org/doi:10.5061/dryad.8q153).