

No correlation between neonatal fitness and heterozygosity in a reintroduced population of Père David's deer

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Abstract Considering the severe impacts of genetic bottlenecks and small numbers of founders in populations of reintroduced animals, it is necessary to study inbreeding and its effect on fitness in species of conservation concern. Père David's deer is one of few large mammal species extinct in the wild but safely preserved in captivity. Its specific background gives us the opportunity to study the relationships between heterozygosity and neonatal fitness in relocated populations. We employed five microsatellite loci to explore heterozygosity-fitness correlations in a population of Père David's deer at the Beijing Milu Ecological Research Center. We observed associations between microsatellite-based variables *sMLH*, *IR*, *MD*² and *HL*, and two components of fitness expressed early in life (birth weight and the neonatal mortality of 123 Père David's deer calves born over six consecutive years). We found that neonatal mortality was $19.1\% \pm 7.6\%$, not higher than the 19% or 18% reported in other ungulates. The heterozygosity of calves was not associated with neonatal mortality, nor birth weight. Our study implies that low genetic variability of microsatellite loci has no overt effect on birth weight and neonatal mortality in reintroduced populations of Père David's deer [*Current Zoology* 59 (2): 249–256, 2013].

Keywords *Elaphurus davidianus*, Birth weight, Neonatal mortality, Founder effect, Inbreeding depression

The conservation of endangered species often requires the reintroduction of captive-bred individuals into the wild (Haig et al., 1990; Seddon and Soorae, 1999; Friar et al., 2001). A loss of heterozygosity in small populations has a deleterious effect on fitness and the genetic consequences of a small number of founders or genetic bottlenecks are concerning (Frankham, 1995; Hedrick and Kalinowski, 2000; Reed and Frankham, 2003). For conservation breeding of endangered animals with very small founder population sizes, inbreeding depression is a critical phenomenon that can increase the risk of extinction (Coulson et al., 1998; Kalinowski and Hedrick, 1998; Hedrick and Kalinowski, 2000; Slate and Pemberton, 2002; Pemberton, 2004; Charpentier et al., 2005). If deleterious recessive alleles are segregating in a population, or if heterozygote advantage is a general phenomenon across the genome, increased homozygosity due to inbreeding will reduce fitness (Coulson et al., 1998; Frankham, 1999; Slate and Pemberton, 2002; Charpentier et al., 2005). However, studies on the influence of inbreeding on overall fitness in natural or semi-natural populations are rare due to the difficulty in establishing multigeneration pedigrees. For

this reason, it is uncommon to calculate inbreeding coefficients in natural or semi-natural populations, particularly for organisms with longer life expectancies (Coltman et al., 1998). Alternatively, heterozygosity-fitness correlations (HFCs) at non-coding genetic markers are commonly assumed to reflect heterozygosity effects on genome-wide distributed genes on different fitness components, like body size, survival rate, fecundity, mating success, physiological parameters and parasite resistance (Coltman et al., 1998). Microsatellites have become the marker of choice in many population genetic studies owing to their abundance and high variability in most eukaryote genomes (Coltman et al., 1998; Slate et al., 1998; DeSalle and Amato, 2004; Garner et al., 2005). Microsatellite-based variables offer measures of individual genetic diversity, such as multi-locus heterozygosity (*MLH*), internal relatedness (*IR*), mean d^2 (*MD*²), and the homozygosity by locus *HL* (Aparicio et al., 2006; Coltman et al., 1998; Charpentier et al., 2005; Coltman and Slate, 2003; Coulson et al., 1998; Hedrick et al., 2001; Slate and Pemberton, 2002).

Père David's deer is one of few large mammal species extinct in the wild but safely preserved in captivity

(Beck and Wemmer, 1983). According to fossil records Père David's deer was once widely distributed across East Asia, but became extinct in the wild in the late 19th century (Ohtaishi and Gao, 1990; Cao, 1993). The last herd of Père David's deer in the Nanyuan Royal Hunting Garden were slaughtered in Beijing by an invading army in 1900. Luckily, a few individuals had been introduced into Europe previously: during the last decade of the 19th century the 11th Duke of Bedford collected the last 18 Père David's deer from Berlin, Paris and Antwerp to form a breeding herd at Woburn Abbey, England (Sowerby, 1949; Jones and Manton, 1983). In 1985 China established the first breeding herd of Père David's deer from a reintroduction program in Beijing. Since then, reintroduced Père David's deer populations have been growing steadily (Yang et al., 2003) and now this species appears to be safe from extinction and individuals have been transferred to zoos or safari parks around the world (Jiang et al., 2000; Jiang, 2011).

Genetic diversity in Père David's deer populations is low. No polymorphism has been found based on plasma protein electrophoresis analysis (Ryder et al., 1981). A single mtDNA D-loop haplotype has been found in reintroduced Père David's deer populations, and genetic variability of microsatellite loci is extremely low in China, in which 2-4 alleles in each locus are present (Zeng et al., 2007; Wu et al., 2008). However, the average level of inbreeding in Père David's deer from zoological gardens is relatively low (0.028) according to data from the International Species Information System (ISIS). In general, the effect of inbreeding on life expectancy was small for the above mentioned inbreeding level (Sternicki et al., 2003).

Considering the severe genetic bottleneck and the small number of founders it is necessary to study inbreeding depression in Père David's deer populations for genetic management. The Beijing Père David's deer population has become the main source of Père David's deer for zoos and safari parks across China. For example, 91 Père David's deer were relocated to form the largest reintroduced population of Père David's deer in Shishou Tianezhou Nature Reserve in Hubei province in the early 1990s. Here, we focus on the Père David's deer population at the Beijing Milu Ecological Research Center. The main aim of this paper is to study the heterozygosity-fitness correlation (HFC) in this reintroduced Père David's deer population. In particular, we explore correlations between microsatellite-based variables and two components of neonatal fitness expressed early in life history: birth weight and neonatal mortality.

1 Materials and Methods

1.1 Study population

The deer population is located at the Beijing Milu Ecological Research Center (39° 07'N, 116° 03'E), and was established with 38 deer from Woburn Abbey, UK in 1985 and 1987. The mean annual temperature of the area is 13.1°C, ranging from -3.4°C in January and 26.4°C in July. Dominant grasses are *Eleusine indica*, *Eragrostis cilianensis*, *Digitaria sanguinalis* and *Setaria viridis*. The deer graze freely on natural vegetation in a 60 ha area in summer and autumn and rely on supplementary feed in winter and early spring. Due to the limited capacity of the habitat, the center has exported deer to zoos, safari parks and reserves across China while maintaining around 120 deer inside the park since 1998 (Zeng et al., 2007).

1.2 Collecting samples

The protocol of handling animals met the requirements of the Chinese Wildlife Management Authority and the Experimental Animal Ethical Committee of the Institute of Zoology, Chinese Academy of Sciences (IOZ-2006). All animals were cared for in accordance with the principles and approval of the Beijing Milu Ecological Research Center. During the calving season from early April to late May, searching for new-born calves was conducted twice a day (early morning and dusk). Each calf was caught during the first day after birth and then weighed, sexed and marked with four-position ear notches by deer keepers (Carnio and Killmar, 1983). All handling lasted less than 10 min; we ensured that calves were led away by their mothers after manipulation. We collected the pieces of tissue from the ear notches and these were frozen below -18°C in a refrigerator until laboratory analyses. Neonatal mortality was defined as the death of a calf within one month after birth. A total of 123 new born Père David's deer calves were sampled from 1999 to 2005.

1.3 DNA isolation and genotyping

Total genomic DNA was isolated from ear tissue using standard organic extraction procedures (Sambrook et al., 1996). Five microsatellite markers (*TI72*, *TGLA10*, *TI93*, *BM1225*, and *BM757*) were used in genotyping (Zeng et al., 2007). Multiplex PCR were carried out with each forward primer fluorescently labeled with TAMRA, 6-FAM, or HEX. Amplifications included about 25 ng of total DNA in 1× amplification buffer in a 25 µl volume, with 3.0 mM MgCl₂, 0.2 mM dNTPs, 0.4 µM of each primer, 0.1 mg/ml of BSA and 1.0 unit Ex *Taq* Hot Start polymerase (Takara). The be-

ginning annealing temperature was 60°C, followed by a touchdown for 1°C at each cycle until 50°C at which it ran for 30 cycles. To avoid allele drop-out, multiplex PCR and genotype detection of each sample were independently repeated three times. PCR products were electrophoresed using an ABI PRISM 377 DNA Sequencer (Applied Biosystems/Perkin Elmer). The fluorescently labeled DNA fragments were analyzed and scored using GeneScan v.3.7 (Applied Biosystems/Perkin Elmer).

1.4 Molecular analysis and sibling assignments

The MS EXCEL add-in MS_TOOLS v.3 toolkit was used to format microsatellite data setting and to create input files for subsequent analyses (Park, 2001). Loci characters like number of alleles per locus; frequency of heterozygotes at each locus, Deviation from Hardy–Weinberg equilibrium (HWE) was tested based on 10,000 Markov chain iterations per batch as implemented in the program Genepop on the web 4.0.10 (Raymond and Rousset, 1995; Rousset, 2008); linkage disequilibrium between markers was tested by the LinkDos program (Garnier-Gere and Dillmann, 1992).

Père David's deer is polygynous. One successful stag may control and mate with a group of hinds in a mating season (Jiang et al., 2004). A hind gives birth to several calves in consecutive fertile years. If calves share the same parent, the effect of full sib on survival and body character could not be ignored. COLONY (Wang, 2004; Wang and Santure, 2009) implements a maximum likelihood method described into assign/infer parentage and sibship among individuals using their multi-locus genotypes. We used COLONY to estimate full-sib relationships among calves, and assigned the colony that a calf belonged to.

1.5 Individual heterozygosity

Heterozygosity at five microsatellite loci was calculated for all calves ($n=123$). The simplest of these was multilocus heterozygosity (*MLH*), estimating the proportion of loci that are heterozygous: if an individual was heterozygous at a locus, it was scored as '1' and if homozygous as '0'. Standardized multilocus heterozygosity (*sMLH*) is the ratio of the heterozygosity of an individual to the mean heterozygosity of those loci at which the individual was typed. This measure avoids potential bias that may be introduced by individuals being untyped at particular loci. The mean across all loci scored was then taken (Coltman et al., 1998; Coulson et al., 1998; Slate and Pemberton, 2002).

The second attempt was to estimate the relatedness of an individual's parents by using the extent of allele

sharing relative to randomness. This has been achieved in the measure known as internal relatedness (*IR*), and was calculated as:

$$IR = (2H - \sum f_i) / (2N - \sum f_i)$$

Where: H is the number of homozygous loci, N is the number of loci genotyped, and f_i is the frequency of the i_{th} allele contained in the genotype. The more an individual is genetically diverse, the more *IR* will be negative (Charpentier et al., 2005; Amos et al., 2001).

Our third approach was to calculate mean d^2 (MD^2) (Coltman et al., 1998; Coulson et al., 1998) as the squared distance in repeat units between the two alleles an individual had at a microsatellite locus, averaged over all loci at which an individual was scored.

$$MD^2 = \sum_{i=1}^n \frac{(i_a - i_b)^2}{n}$$

Where: i_a and i_b are the lengths in repeat units of alleles a and b at locus i and n is the number of typed loci (Coulson et al., 1998; Frankham, 1999). Then its standardized measure, the *sMD*² is that MD^2 was standardized and divided by the variance of d^2 over individuals at the i_{th} locus, thereby ensuring that each locus contributed equally (Hedrick et al., 2001).

The fourth estimate of HFC in this study was the Homozygosity by locus (*HL*) that Aparicio et al. (2006) developed:

$$HL = \sum E_h / (\sum E_h + \sum E_j)$$

Where E_h and E_j are the expected heterozygosities of the loci that an individual bears in homozygosis (h) and in heterozygosis (j) respectively (Aparicio et al., 2006). Except for mean d^2 , other HFC metrics were calculated using a R function (GENHET) (Coulon, 2010).

1.6 Data analysis

All data were checked for normality with one sample Kolmogorov-Smirnov tests using *ks.test* in R (Development Core Team, 2012). Data which were not normally distributed were transformed before parametric statistical analyses. Colony ID was included as a random effect to control for possible nonindependence of living success within colonies. We used Fit Mixed-Effects Models (*Imer*) in R to fit the model and examine the relationship between birth weight and *sMLH*, *IR*, MD^2 or *HL*. Neonatal mortality is a binary variable and was analyzed using Generalized Linear Models (*glmer*) in R to fit the logistic regression models, in which birth weight was considered a covariate and each HFC were included independently. The sex of calves was included as a fixed factor in linear but also in logistic regression models. We conducted analysis of variation models when estimating

differences between sexes in birth weight and for the association between birth weight and probability of mortality. R was also used to conduct t tests, generate graphs and to look for correlations between HFC estimators. Data are presented as mean \pm SD and the level of statistical significance for all statistical tests was set at $P < 0.05$.

2 Results

Calf mortality of $19.1 \pm 7.6\%$ observed during the study period was normally distributed ($P = 0.14$). This value is not significantly different from the rates of 19% ($t=0.57$, $P=0.95$) and 18% ($t=0.429$, $P=0.68$) previously reported (Linnell et al., 1995; Coulson et al., 1998) using a one-sample t test. Birth weights of 12.2 ± 1.76 kg were not normally distributed. There was no significant difference between the birth weight of male calves and that of female calves using a t test (61 males versus 62 females, $t=-1.0228$, $df=121$, $P=0.309$). Sex had no significant influence on calf survival rate and no difference was detected in the birth weight of calves from different colonies ($F=1.509$, $P=0.0781$).

Observed heterozygosity at each locus ranged from 0.48–0.63, and allelic diversity was low (Table 1). There was no evidence of genotypic linkage disequilibrium at

Table 1 Polymorphism characteristics of microsatellite loci used to type Père David's deer calves born from 1999–2005

Locus name	N_A	H_o	H_e	P_{HWE}
T172	0.6650	0.51	0.49	0.6809
TGLA10	0.3492	0.54	0.48	0.1880
T193	0.3166	0.61	0.63	0.2791
BM1225	0.5334	0.49	0.50	0.8545
BM757	0.5020	0.54	0.50	0.3699

N_A : Maximum likelihood estimation of null allele frequency. H_o : Observed frequency of heterozygotes. H_e : Expected frequency of heterozygotes. HWE: Significance level of deviation in expected genotype frequencies from Hardy-Weinberg expectation calculated by Fisher's exact test (Raymond and Rousset, 1995).

any pair of loci (all $P < 0.05$). All multilocus heterozygosity estimates were strongly inter-correlated ($P < 0.01$, $r > 0.88$).

Using the colony as a random factor and sex as a fixed factor, birth weight and neonatal mortality were uncorrelated ($z=1.943$, $P=0.0521$). We constructed separate logistic mix models of calf survival and their MLH , IR , MD^2 or HL , in which birth weight was treated as a covariate. The frequency distributions of these variables are shown in Figure 1. No heterozygosity meti-

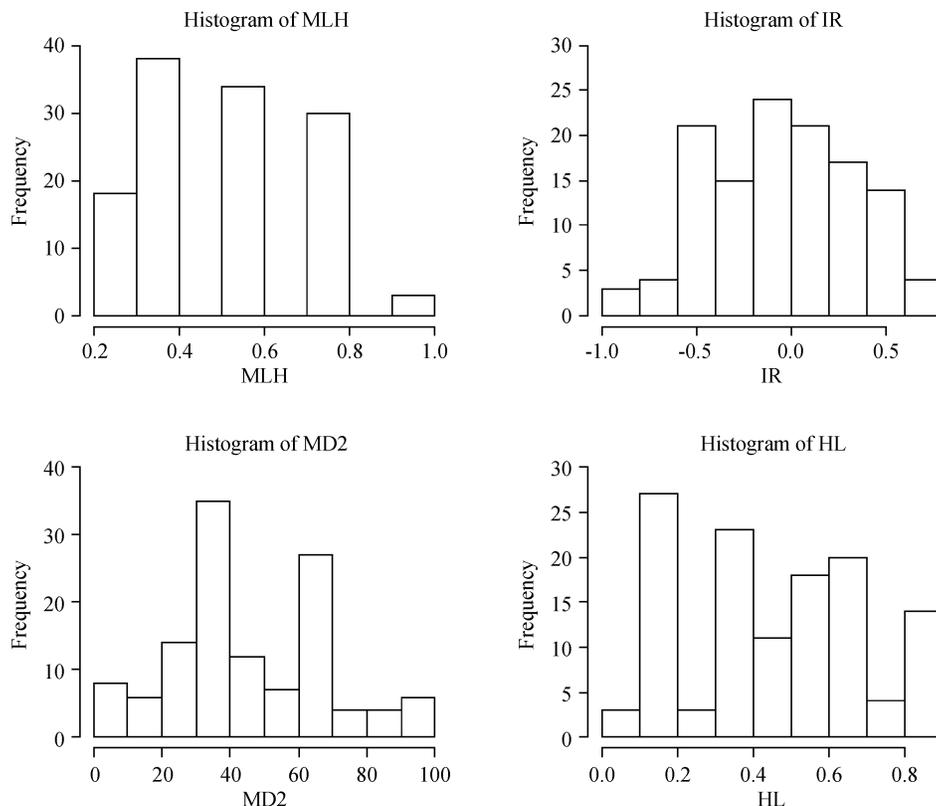


Fig. 1 Frequency distributions of standardized multilocus heterozygosity (MLH), internal relatedness (IR), standardized mean d^2 (MD^2) and the homozygosity by locus (HL) of 123 Père David's deer calves in a population in Beijing

ric was significantly correlated with neonatal mortality (Table 2). When fitted alone in a simple linear regression model, heterozygosity measures and birth weight were not significantly correlated (Table 2). All modeling considered colony a random factor.

Table 2 Correlation between individual heterozygosity and birth weight and neonatal mortality

	Mean value	Birth weight (with colony as random factor)		Neomortality (with colony as random factor, and birth weight as covariate)	
		AIC	t	z	P
<i>sMLH</i>	0.5382	360.5	-0.410	-1.080	0.2802
<i>IR</i>	-0.03195	360.8	0.357	0.948	0.3433
<i>MD</i> ²	46.11	359.9	-0.976	-1.273	0.2029
<i>HL</i>	0.4579	360.7	0.410	0.722	0.4705

3 Discussion

Inbreeding may greatly reduce average individual fitness and erode genetic diversity due to random genetic drift in small populations and thus reduce the ability for individuals to adapt to changing environments (Lande, 1988). Père David's deer in China has survived a number of severe genetic bottlenecks: (1) when the deer was transported from China to Europe in the 19th century; (2) when deer were collected to form the first breeding population at Woburn Abbey; (3) when deer were transferred to Whipsnade Wild Park and other UK zoos; and (4) when reintroduced to China in the 1980s. Due to these bottlenecks, as well as their polygynous mating system and matriarchal social structure, genetic variation in Père David's deer may be deficient. The Beijing population is the first established reintroduced population and the source of most Père David's deer populations in China. Due to the small size of the Beijing center, the deer population has been maintained at a finite size for a decade and the population faces a high rate of genetic drift due to inbreeding. However, there is little evidence of inbreeding depression or heterozygosity-fitness correlation expressed in calves in this population thus far. Neonatal calf mortality was not high compared to the average neonatal mortality of 19% for ungulates living in habitats that lack predators (Linnell et al., 1995), and not higher than the 18% seen in red deer calves *Cervus elaphus* in an island population under impact of predation (Coulson et al., 1998). Average inbreeding depression on longevity was small for Père David's deer calves born from 1947–2000 in zoological gardens (Sternicki et al., 2003). These comparisons show that this trait is not subject to inbreeding depres-

sion in Père David's deer.

No correlation was found between measures of individual genetic markers and neonatal fitness in the population of Beijing Père David's deer studied here. Previous studies of wild vertebrate populations have reported positive associations between genetic variation measured at microsatellite loci and fitness. In wild harbor seal *Phoca vitulina* pups, birth weight was positively influenced by maternal age, pup sex, and either *MD*² or *MLH* in separate multiple regression models (Coltman et al., 1998). *IR* is also an important determinant of pre-weaning pup survival in grey seal *Halichoerus grypus* pups (Bean et al., 2004). Low *MD*² has been associated with low birth weight and low neonatal survival in red deer calves (Coulson et al., 1998; Slate et al., 2000) and in roe deer *Capreolus capreolus* juveniles (Silva et al., 2008), which may suggest a possible outbreeding advantage.

However, previous studies have found no significant relationship between individual heterozygosities and traits of fitness like resistance to parasites or hatching success (Côté et al., 2005; Ortego et al., 2007; Ortego et al., 2010). This was true for a study of 1070 Antarctic fur seal pups born on Bird Island, South Georgia (Hoffman et al., 2006). It has been argued that this universally positive result may be misleading because negative results frequently go unpublished (Coltman and Slate, 2003; Hoffman et al., 2006). Meta-analyses of published and unpublished correlations between phenotypic variation and two measures of genetic variation at microsatellite loci (*MLH* and *MD*²) revealed that the strength of these association is generally weak (Coltman and Slate, 2003).

Neonatal mortality and birth weight of Père David's deer calves are not associated with genetic diversity. It seems that low genetic variability of microsatellite loci has no effect on fitness of reintroduced Père David's deer populations in China. The absence of relationship between fitness-related traits and multilocus heterozygosity may be a consequence of a progressive purge of recessive deleterious alleles in the reintroduced Père David deer population (Zeng et al., 2007). It is less likely that inbreeding depression occurs in other Père David's populations, because the Beijing population has the smallest effective size and is subject to the heaviest inbreeding stress among reintroduction populations in China (Zeng, 2007). China hosts more than half of all Père David deer in the world and the population genetic status of those reintroduced deer in the country may reflect the situation of all other Père David deer

populations. Though we did not find any correlation between heterozygosity and neonatal fitness in reintroduced Père David's deer, the time span since the reintroduction may be too short to draw any absolute conclusions.

Which metrics of heterozygosity best reflect HFC over time is still being debated (Tsitrone et al., 2001; Coltman and Slate, 2003; Neff, 2004; Aparicio et al., 2006; Silva, et al., 2008; Chapman et al., 2009; Malo and Coulson, 2009). *MLH* is likely to correlate with recent inbreeding (Coltman and Slate, 2003; Neff, 2004). *IR* may underestimate the heterozygosity of individuals carrying rare alleles (Aparicio et al., 2006). *MD*² may be more appropriate than *MLH* for measuring divergence times over longer time scales and thus for detecting outbreeding depression or heterosis (Silva, et al., 2008). In a panel of 71 microsatellite loci in wild red deer *Cervus elaphus*, Coulson et al. (1998) suggested that heterozygosity-based measures outperform mean *d*²-based measures. By weighing the contribution of each locus to homozygosity, *HL* better correlates with genome-wide homozygosity and inbreeding coefficient in open populations (Aparicio et al., 2006; Ortego et al., 2007; Ortego et al., 2010). To reveal possible aspects of HFC, we used all four metrics and found correlation among the metrics is significant, as has been shown in previous studies (Coltman et al., 1998; Slate and Pemberton, 2002; Hoffiman et al., 2006).

Our study used five polymorphic microsatellites to estimate the heterozygosity of each individual. This level falls below the typical level of number of loci used in HFC studies and may result in low power to detect associations (Slate and Pemberton, 2002; Balloux et al., 2004; Chapman et al., 2009; Ortego et al., 2007; Ortego et al., 2010). Furthermore, the availability of using microsatellite marker to reveal HFC is uncertain (Silva et al., 2008; Chapman et al., 2009; Malo and Coulson, 2009). HFCs may be identified under three possible causes: inbreeding depression, overdominance, or associated overdominance. It is thought to reflect the inbreeding level of an individual in genome wide genetic diversity (Slate et al., 2004), since microsatellite markers are usually located in introns, do not suffer direct selection, and seldom link with overdominant or dominant loci (Silva et al., 2008; Malo and Coulson, 2009). However, microsatellite markers may be poor indicators and not accurately reflect the underlying genomic diversity at a population level (Väli et al. 2008).

Even if molecular measures are useful tools to detect inbreeding depression, this study did not detect in-

breeding depression and the methods cannot be used to rule out the existence of depression. Due to the short period since population establishment, a small population may not have sufficient time to accumulate spontaneous deleterious mutations over 20 years. The status of a population will change from vulnerable to extinction via a mutational meltdown in approximately 100 generations (Lynch et al., 1995). We may have to wait to see how the reintroduced Père David deer respond to plausible environmental change and natural catastrophes in the future. Thus, genetic diversity is still critical for animals like Père David deer to cope with the environment. The question remains, how to best slow down genetic erosion in Père David deer? We suggest setting up a comprehensive genetic archive for Beijing Père David's deer using the data on hand. When establishing new Père David deer populations, the genetic background of each founder should be explored to make certain genetic variations are preserved in both source and relocated populations.

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