Current Biology *16*, 612–617, March 21, 2006 ©2006 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2006.02.059

Report

Reduced Heterozygosity Depresses Sperm Quality in Wild Rabbits, *Oryctolagus cuniculus*

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Summary

When close relatives are forced to reproduce, the resulting offspring inherit above average homozygosity and reduced fitness [1, 2]. Biologists now recognize inbreeding depression in the wild [3–5], a phenomenon that will probably increase as natural populations become depleted and fragmented. Inbreeding depression is most commonly expressed as compromised fertility and embryogenesis [4], but actual mechanisms remain poorly understood, especially for wild populations. Here, we examine how reduced heterozygosity influences spermatozoal and gonadal traits in wild rabbits (*Oryctolagus cuniculus*) sampled across the United Kingdom. By using a suite of 29 microsatellite markers (analyzed to confirm representation of individual heterozygosity across our sample), we found a significant negative relationship between heterozygosity and the production of normal sperm; the relationship was significant both between (n = 12) and within (n = 91 [total males], 42 [island], 49 [mainland]) populations. Reduced heterozygosity was also associated with decreased testis size across males (n = 112), but no relationship was seen at the population level, suggesting environmental confounds. Our results show, for a wild mammal, that inbreeding is associated with decreased sperm quality, confirming suggestions of links between inbreeding and elevated sperm abnormalities in rare felids [6–8]. These findings could explain why inbreeding depression so frequently arises via compromised fertility and embryogenesis [4].

Results and Discussion

The relationship between depleted genetic variation and inbreeding depression has been recognized since the 1800s under domestic breeding [9]. However, evidence for inbreeding effects in the wild was previously doubted [10], despite evidence that taxa under inbreeding risk have evolved recognition, mating, and dispersal strategies that avoid breeding with close relatives [11]. Biologists have recently described inbreeding effects in wild populations, where fitness depression may be accentuated by natural selection, and inbreeding even causing local extinctions [5]. Although now generally recognized, it has been particularly challenging to identify the specific mechanism leading to fitness depression under inbreeding in free-living populations because (1) inbred individuals will be much rarer for study, (2) wild taxa are difficult or impossible to experimentally manipulate, and (3) suitable methods for objectively measuring heterozygosity have only recently been developed. Despite these problems, it is essential to examine inbreeding depression under the selective conditions of the natural environment, in order to identify and understand responses to reduced heterozygosity [4].

We use a molecular measure of degree of inbreeding as average heterozygosity across 29 microsatellite loci for each individual male rabbit. Previous analytical and metanalytical studies suggest that mean heterozygosity is a better measure of genetic variability for inbreeding studies than d^2 [12, 13]. Although the relationship between the microsatellite loci and the genes coding for inbreeding depression is usually not known, molecular measures overcome obvious constraints of using pedigree-based methods to measure inbreeding in natural populations and the barriers to tracking paternity in mobile, wild lineages. Furthermore, the higher mutability of microsatellites means that these markers are likely to be most suitable for measuring genotype:fitness correlations within populations that have a more recent evolutionary history [14], such as has occurred through the colonization of European rabbits into the UK since Roman times [15]. Of our 29 loci, ten have been mapped (A.K.S., L.W., and M.J.G.G., unpublished data) and cover at least six different chromosomes. Three loci belong to one linkage group, and two loci show consistent linkage disequilibrium (A.K.S., L.W., and M.J.G.G., unpublished data); however, no physical linkage appears to exist between any of the other 24 loci.

An important assumption to test when using microsatellite loci to measure individual heterozygosity is whether sufficient loci are scored to represent genome-wide heterozygosity. Recent empirical and theoretical approaches show that loci numbers required may be far in excess of that usually analyzed [12, 16]. A method to test formally whether loci number represents individual heterozygosity is to randomly divide the loci into two groups, calculate mean individual heterozygosity for either group across the sample, and determine whether the two measures of mean heterozygosity (each derived from half of the loci suite sampled) are correlated across individuals [12]; this process is repeated by randomized placing of the loci within either of the two groups. Across 91 males (for which sperm data were collected), we find significant correlations between the two averages of mean heterozygosity, suggesting

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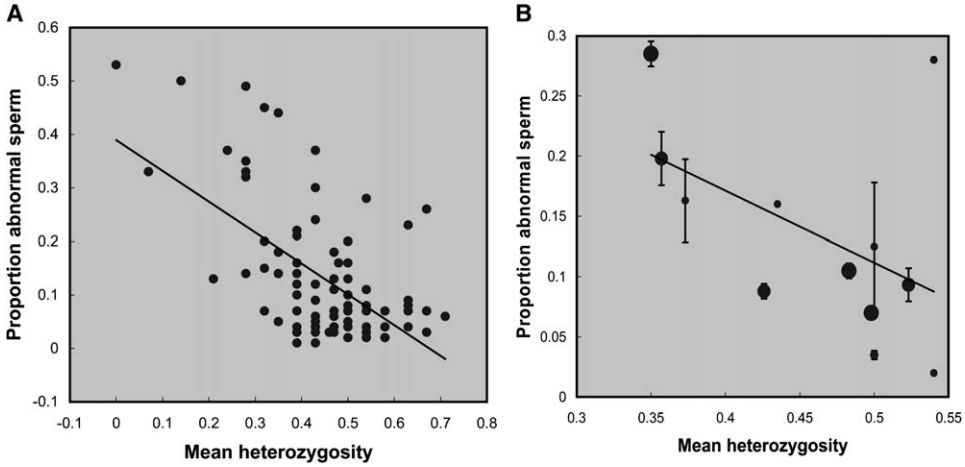

Figure 1. Across-Individual and Across-Population Relationships between Average Heterozygosity and Sperm Abnormality in Wild Rabbits

(A) The proportions of abnormal sperm (arcsine transformed) across 91 individual male rabbits show a significant negative relationship ($R^2 = 0.33$ [±0.07], $p < 0.001$, $Y = -0.58X + 0.39$) with average heterozygosity. (Heterozygosity is scored across 29 genome-wide microsatellite loci and % sperm abnormalities scored from screens of 81 to 771 [average 320] sperm per male.) Removal of the three potential outliers preserves the relationship ($R^2 = 0.19$, $p < 0.001$, $n = 88$). A similar relationship exists for % decapitated sperm, but not for the incidence of cytoplasmic droplets.

(B) Average proportions of abnormal sperm (arcsine transformed with standard errors) show a significant relationship with average heterozygosity across 12 sampling sites (least squares regression weighted by sample sizes: $R^2 = 0.7$, $p < 0.001$; weighted by reciprocal of the variance: $R^2 = 0.58$, $p = 0.0039$; removal of the outlier [represented by a single male]: $R^2 = 0.73$, $p = 0.001$, $n = 11$). Large markers indicate means derived from >5 males, small markers <5 males. Sampling sites and actual sample sizes identified in Table S1.

high repeatability of our heterozygosity measures. Repeat analysis over 100 permutations, randomizing the representation of each locus contributing to either half's mean in each run, shows that the average correlation between the heterozygosity means for the two loci groups was $R = 0.31$ and $p = 0.002$ ($n = 100$ iterations with means from 91 males, SD = 0.062). Only two out of 100 permutations generated correlations where $p > 0.05$ (range: $R = 0.195$, $p = 0.061$ to $R = 0.5$ and $p < 0.0001$). Although recent theoretical and empirical analyses have concluded that the power to represent mean heterozygosity will be low, even with large numbers of loci [12, 16], the wide variance in heterozygosity values across our 91 male sample, suggesting true inbreeding and structured populations, may allow the UK wild rabbit system to generate a representative measure of average heterozygosity via these 29 loci; note that linkage does not explain these relationships (A.K.S., L.W., and M.J.G.G., unpublished data).

By using mean heterozygosity, we explored associations with phenotypic traits adopting a hierarchical analysis approach that analyzes both across populations and individuals within and across populations. We adopt this broad-scale approach to determine that relationships occur both at the level of the population (and hence not confounded by [1] potential environmental variation and/or [2] statistical nonindependence of individuals) and also across individuals (because [1] relatively recent colonization of the UK by rabbits [15], and [2] previous work [18] demonstrating extremely fine-scale genetic structuring in UK rabbits over short spatial scales both make it difficult to define what constitutes a "population" for analysis). One obvious signal of potential environmental variation was the 2-fold range in adult male body weight across our sample; however, we found no relationship between heterozygosity and male size ($R_{sp} = 0.145$, $p = 0.13$, $n = 112$; note that this relationship did not produce normally distributed residuals [in contrast to the parametric relationships], hence the use of a nonparametric Spearman rank correlation). Temporal variation in sampling was also not a confound variable: sampling date showed no association with heterozygosity ($R = 0.06$, $p = 0.51$, $n = 112$).

Proportion data were arcsine transformed. In every linear regression analysis, we had first established that residuals were normally distributed, and therefore fitted assumptions of parametric analysis. Because of unequal sample sizes in different populations, and since there was one obvious outlier (which was driven by a single male), we used weighted means in performing regressions across population means, weighting both by sample size and by the reciprocal of the population mean's variance (using the mean value itself for the two populations represented by single males) [19, 20]. Standard linear regression models assume that variance is constant within the population under study. As this is not the case in our cross-population analysis because of unequal sample sizes, we can instead compute the coefficients of a linear regression model by using weighted least squares regression, such that the more precise observations (i.e., those with larger sample sizes and/or lower variance) are given greater weight in determining the regression coefficients. The weight estimation procedure in SPSS allows a quantifiable weighting (e.g., sample size or a measure of variance) to be applied to different means and allows fitting of the data on the basis of the weights [19, 20].

The proportion of abnormal sperm showed a significant negative association with mean heterozygosity across individual males ($R^2 = 0.33$ [±0.07], $p < 0.001$, $F_{1,90} = 44.1$, $n = 91$, $Y = -0.58X + 0.39$; Figure 1A). A similar significant relationship existed across the 12

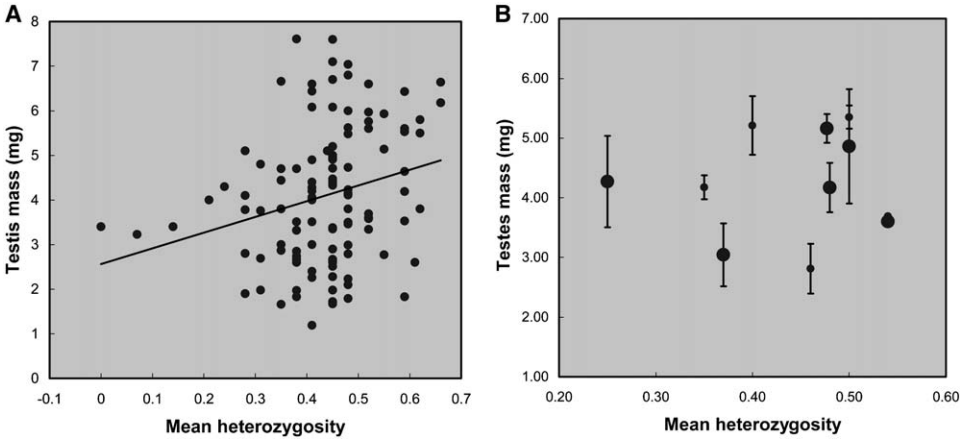

Figure 2. Across-Individual and Across-Population Relationships between Average Heterozygosity and Testis Size in Wild Rabbits

(A) Testis mass increases with heterozygosity across 112 males (R^2 = 0.06 [±1.49], p = 0.008, Y = 3.52X + 2.56). Controlling for potential allometry in a multiple regression reveals a similar significant relationship between heterozygosity and testis weight/body weight combined ($F_{2,109}$ = 3.78, p = 0.026; note that body mass does not correlate with heterozygosity: R_{sp} = 0.145, p = 0.13, n = 112).

(B) Average testis mass (with standard error bars) shows no relationship with mean heterozygosity across 12 populations (weighted means regression: R^2 = 0.02 [±0.874], p = 0.2, n = 12). Large markers indicate means derived from >5 males, small markers <5 males. Sampling sites and actual sample sizes identified in Table S1.

population means (Figure 1B), whether we weighted the regression analysis by sample size (R^2 = 0.69 [±0.13], p < 0.001, $F_{1,10}$ = 22.1, n = 12, Y = −1.0X + 0.58) or the reciprocal of each population mean's variance (R^2 = 0.58 [±0.59], p = 0.0039, $F_{1,10}$ = 13.91, n = 12, Y = −1.03X + 0.55). We also checked this relationship without the outlying population on Rathlin Island (derived from a single male) by using standard linear regression, and we found a significant relationship across 11 populations (R^2 = 0.73 [±0.04], p = 0.001, $F_{1,9}$ = 24.3, n = 11). We further explored this relationship, controlling for potential differences between island and mainland rabbits: across island males, the relationship between heterozygosity is strongly negative (R^2 = 0.44, p < 0.001, $F_{1,41}$ = 31, n = 42, Y = −0.46X + 0.31), while across mainland males, the relationship, though significant, is weaker (R^2 = 0.08, p = 0.047, $F_{1,48}$ = 4.1, n = 49, Y = −0.21X + 0.186). The stronger relationship in the lower range of heterozygosities is further illustrated by one single island population (the Isle of May: R^2 = 0.49 [±0.06], p = 0.008, $F_{1,12}$ = 10.5, n = 13, Y = −0.47X + 0.33) compared with one comparable mainland population showing no such relationship (Blyford Estate, Suffolk: R^2 = 0.006, p = 0.69, $F_{1,28}$ = 0.17, n = 29). It is clear from Figure 1A that phenotypic effects on sperm abnormality appear to occur in the lower range of heterozygosities.

We also find similar negative relationships between heterozygosity and proportion of decapitated sperm across 12 populations (R^2 = 0.64 [±0.04], p = 0.002, $F_{1,11}$ = 17.43, n = 12, Y = −0.71X + 0.39) and 91 males (R^2 = 0.33 [±0.07], p < 0.001, $F_{1,90}$ = 44.12, n = 91, Y = −0.4X + 0.25; note that % decapitate is measured separately and does not contribute to % abnormality). No relationship existed between heterozygosity and proportion of sperm carrying a cytoplasmic droplet across 12 sites (R^2 = 0.20, p = 0.144) and 91 males (R^2 = 0.04, p = 0.06). Note that the cytoplasmic droplet trends with heterozygosity are positive.

We found a significant positive relationship between testis weight and mean heterozygosity across all males, so that more inbred males had smaller testes (R^2 = 0.06 [±1.49], p = 0.008, $F_{1,110}$ = 7.32, n = 112, Y = 3.52X + 2.56; Figure 2A). Because of allometry, we also entered body mass and testes weight into a multiple regression, and the relationship between heterozygosity and testes weight remained ($F_{2,109}$ = 3.78, p = 0.026; note that body mass does not correlate with heterozygosity). However, no relationship between heterozygosity and testes weight existed at the population mean level (means weighted by sample size: R^2 = 0.15 [±0.087], p = 0.2, n = 12; means weighted by reciprocal of the variance: R^2 = 0.014 [±1.0], p = 0.71, n = 12), suggesting interpopulation differences in testis weight that might be driven by environmental variation.

Our results therefore provide evidence from the natural environment that inbreeding leads to compromised sperm quality. Figures 1A and 1B show that decreasing heterozygosity is significantly associated with increasing proportions of sperm abnormalities across male rabbits. A similar association exists for the proportion of decapitated sperm, and the phenomenon of "easily decapitated sperm" under standard micromanipulation is associated with infertility in humans [17]. Figure 1A reveals a predictable skew in the distribution of heterozygosities sampled from wild populations (heterozygosity skew = −0.737 [±0.253 SE], n = 91, p < 0.05). If there are fitness constraints associated with inbreeding [2, 3–5, 11], individuals with high homozygosity will inevitably be rarer than heterozygous conspecifics in wild populations. By investigating a range of 12 island and mainland sampling sites across the UK, we were able to sample a full breadth of heterozygosities (with one extreme individual that was remarkably homozygous at all 29 microsatellite loci). Although island isolation will increase the overall probability of inbreeding, within our sample there is no significant difference between island and mainland

heterozygosities ($t = -1.87$, $p = 0.07$, $n = 42$ [island mean $H' = 0.42$] and 49 [mainland mean $H' = 0.47$] individuals), and we find associations between heterozygosity and sperm abnormality across either island or mainland males. It is clear that the stronger relationship occurs across the lower range of heterozygosities, suggesting that inbreeding depression occurs only when a population becomes more strongly inbred. The comparison between the Isle of May population (which exhibits a wide range of heterozygosities) with the mainland Suffolk population (which shows no significant relationship) illustrates that inbreeding effects on sperm quality appear to occur in the lower heterozygosity range.

Across larger sites without spatial isolation, it is difficult to identify what defines a population in terms of gene flow for rabbits. Wild rabbits in Britain exhibit marked social and territorial behaviors typical of many mammals. In particular, strong patterns of female philopatry lead to fine-scale genetic structuring within rabbit populations, with social groups forming distinct genetic units [18]; hence, genetic inbreeding can occur over very small spatial scales. This genetic segregation over relatively small spatial scales leads to significant variance in heterozygosity within one location. Accordingly, a single sampling site may contain numerous populations with respect to genetic structuring, and this is one reason why we also identify individual males as independent data points for analysis (while checking for the same relationships within and between populations). In addition to this genetic variation over short distances, it is unlikely that environmental variance explains the relationship between heterozygosity and sperm quality since similar associations exist at the level of the population mean. Previous work exploring "environmental" influences on sperm morphometry also suggests a minor influence of environment on individual sperm integrity. In one study where developing male meal moths were protein starved through spermatogenesis, no influence was found on sperm morphology, despite drastic reductions in body size, testis size, and ejaculate sperm number [21].

Sperm abnormality is a widespread phenomenon [22], with fundamental implications for male reproductive success. In humans, elevated proportions of spermatozoal abnormality (typically above 40% nonnormal) are associated with male-factor infertility [23, 24]. In other mammals (including rabbits [25]), males with higher proportions of abnormal sperm show reduced fertility [26]. Why so many species produce abnormal sperm is poorly understood. Proposals that atypical sperm morphologies in vertebrates are adaptive in sperm competition have received no experimental support [27]. Environmental extremes such as exposure to toxins (e.g., [28]) or nutritional deprivation [29] can increase gametic abnormalities, but there is a significant base-level of gamete abnormality in apparently ordinary males of many taxa (e.g., 14%–44% in humans with normal fertility [30]), which can be as high as 90% [6]. Some "maturational" abnormalities, such as the presence of a cytoplasmic droplet, may result from sperm ageing in storage [31], but significant proportions of abnormal sperm result from primary production within the testis and epididymis [32].

The relationships we find provide empirical support to the controversial suggestions of a link between inbreeding in rare felids and sperm quality [6–8]. The endangered Florida panther, for example, shows very low heterozygosity and most of the sperm produced by males (80%–90%) are abnormal [6]. However, suggestions of a link between inbreeding and sperm abnormality are controversial and remain unproven because appropriately controlled comparisons with noninbred conspecifics or relatives across natural populations have not been made [33, 34]. One study suggests a link between sperm abnormality and inbreeding in an ungulate [35], but this work examined a captive population and was found in only one of three species examined.

Spermatozoa may be more prone to revealing inbreeding depression than other traits because spermatogenesis is a complex process demanding precise genetic and physiological control to produce the most specialized of eukaryotic cells [22]. Sperm abnormalities can arise from primary or secondary causes [32]. Increased mutational defects in the male germline or genetic errors in spermatogenesis generate primary abnormalities and could be generated via low genetic diversity that increases the expression of homozygous recessive mutations [36]. Additionally, sperm quality may be one of the first phenotypic traits that are measurably depressed by inbreeding. Most other cell types exist in a tissue matrix or function collectively for the organism's maintenance, so that a proportion of imperfections can be buffered. However, sperm must show sound individual functional morphology to achieve fertilization success, and this individual specialization may make sperm more sensitive to the deleterious genetic effects of inbreeding. Secondary spermatozoal defects arise within spermiogenic development and maturation in the testis and epididymis and could result from general gonadal compromise. We find some evidence for reduced testis size under low individual genetic variability (Figure 2A), but because this relationship did not hold at the population level, we cannot exclude the possibility that this relationship may be driven by environmental variation or statistical nonindependence at the level of the individual male (Figure 2B). However, in the inbred and hypogonadal Florida panther, testis volume is 40% that of noninbred Latin American conspecifics. Experimentally inbred captive mice (*Peromyscus polionotus*) showed reduced testis size [37], and studies of free-living lions indicate a decrease in Sertoli cell proliferation in inbred males, essential for the production of normal competent sperm [38]. Normal functioning of the testis and epididymis in rabbits allows some reabsorption of abnormal sperm [39] so that gonadal disruption under inbreeding could be one route to production of abnormal sperm. Epididymal duct malfunction leads to the production of more abnormal sperm and, in fowl, efferent duct malformation is directly heritable and manifested in males carrying the Sperm Degeneration allele [40]. This condition provides good evidence that profound compromises to testicular development can arise from single gene effects, and therefore could be more commonly expressed under very low heterozygosity.

Whatever the precise mechanisms controlling the production of abnormal sperm, our finding does identify spermatozoal integrity as a likely male candidate for why

reproduction and fertility is so frequently depressed under inbreeding. In a recent review of 46 studies reporting inbreeding in wild populations [4], 43 found evidence for significant inbreeding depression, with the usual compromise to reproduction. Of these 43 studies describing inbreeding depression, 60% identified the route via significantly decreased fertilization, hatching, or germination success [4].

Experimental Procedures

Field Sampling and Dissection

112 reproductively active male European rabbits were sampled from island and mainland sites across the UK during the breeding season (April–August 2002 and 2003). See Supplemental Data available with this article online. Males were culled by licensed hunters, and total body weight and testes weight were recorded (to 0.01 gram) for each individual. For 91 males (representing all 12 sampling sites), cadavers were refrigerated to prevent pathogen build-up and sperm damage, and a mature sperm sample was recovered within a maximum of 12 hr from the distal *vasa deferentia*, immediately dispersed in PBS buffer, and fixed with 2.5% gluteraldehyde. (Time-since-death using these methods was unrelated to sperm abnormality $R = 0.09$, $p = 0.57$, $n = 46$. Similarly, time from fixation to scoring was unrelated to a male's abnormality score, $R = 0.02$, $p = 0.86$, $n = 56$). An ear-clip was taken from each male and preserved in 70% ethanol for subsequent DNA extraction.

Sperm Analysis

All sperm analyses were conducted blind to molecular screening. Sperm morphological analysis was conducted on fixed samples in buffered solution under cover slips by phase contrast microscopy at ×400 magnification. All undamaged sperm were scored continuously in the field of view until the sample had been screened; an average of 320 sperm per male were scored (range 81 to 771—samples with <250 sperm were due to low sperm numbers recovered from the male). Sperm morphologies were scored as per World Health Organization guidelines for human sperm screening [41], with abnormal sperm designations for defects from normality in the sperm head (shape deviations from ovoid, decapitation, microcephalic, acrosomal disintegration, apical protuberance), mid-piece (bent, distorted, proximal or distal cytoplasmic droplet), or flagellum (coiled, bent, split, biflagellate). Decapitate cells were analyzed separately, because of the possibility that decapitation occurred due to sample preparation, although this is a recognized male infertility condition [17]. Cytoplasmic droplets are likely to reflect a stage of spermatogenic maturation [31] and were therefore not designated as morphologically "abnormal" cells for analysis, although we examined relationships with mean heterozygosity.

In addition to sperm morphology, we also conducted detailed measures of sperm morphometry for 64 males. Sperm were air-dried onto flat glass slides (so they presented a perpendicular plane for linear measurement) and measured without coverslips under ×600 magnification by analySIS microscopy image analysis software. Total lengths of 50 sperm were measured for each male.

Molecular Screening

Total genomic DNA was extracted from rabbit skin tissue by means of a Qiagen DNA minkit (Qiagen) following the manufacturer's protocol. PCR amplifications were performed in 11 μl reaction volumes containing the following: 0.45 μM each primer, 1× ABgene PCR Mastermix with 1.5 mM $MgCl_2$ (ABgene), 1 μl DNA of variable concentration and sterile distilled water to total volume. PCR cycling was performed with a MJ Dyad DNA Engine (MJ Research) with the following parameters: initial denaturation at 94ºC for 3 min; 35 cycles of 94ºC for 15 s, 52ºC–65ºC for 15 s, and 72ºC for 15 s; final extension at 72ºC for 30 min. The final extension was performed in order to maximize "A" addition at the 3′ end of PCR products to increase the accuracy of size scoring. Forward PCR primers were labeled with either FAM, NED, PET, or VIC fluorescent labels, and PCR products were run on an ABI3700 automated DNA sequencer with the Genescan-500 LIZ labeled size standard. Fragment lengths were determined with the Genescan and Genotyper software packages (Applied Biosystems). A full list and analysis of the 29 microsatellite loci are detailed elsewhere (A.K.S., L.W., and M.J.G.G., unpublished data). Ten loci have been mapped and cover a minimum of six different chromosomes. Three loci belong to one linkage group, and two loci show consistent linkage disequilibrium (A.K.S., L.W., and M.J.G.G., unpublished data). No physical linkage appears to exist between the remaining 24 loci.

Supplemental Data

The Supplemental Table can be found with this article online at http://www.current-biology.com/cgi/content/full/16/6/612/DC1/.

Acknowledgments

We are extremely grateful to the farmers, wardens, and gamekeepers who provided rabbits. In particular, we received exceptional help from John Buck of Blyford Estate and Alastair Scott of the Uists. We also appreciate the thoughts of colleagues on statistical approaches, especially Jason Kennington and Rob Freckleton. Two referees significantly improved the manuscript, particularly the statistical analysis design. This work was funded by NERC grant NER/B/S/2001/00381 and the Royal Society.

Received: September 23, 2005
Revised: February 9, 2006
Accepted: February 10, 2006
Published: March 20, 2006

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