Comparing molecular measures for detecting inbreeding depression

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Abstract

Correlations between heterozygosity and components of fitness have been investigated in natural populations for over 20 years. Positive correlations between a trait of interest and heterozygosity (usually measured at allozyme loci) are generally recognized as evidence of inbreeding depression. More recently, molecular markers such as microsatellites have been employed for the same purpose. A typical study might use around five to ten markers. In this paper we use a panel of 71 microsatellite loci to: (1) Compare the efficacy of heterozygosity and a related microsatellite-specific variable, mean d^2, in detecting inbreeding depression; (2) Examine the statistical power of heterozygosity to detect such associations. We performed our analyses in a wild population of red deer (*Cervus elaphus*) in which inbreeding depression in juvenile traits had previously been detected using a panel of nine markers. We conclude that heterozygosity-based measures outperform mean d^2-based measures, but that power to detect heterozygosity-fitness associations is nonetheless low when ten or fewer markers are typed.

Introduction

The role of inbreeding depression has received considerable attention in areas as diverse as the evolution of dispersal and mating systems, conservation biology, animal and crop production and the maintenance of genetic variation (Charlesworth & Charlesworth, 1987; Thornhill, 1993). Under either of the main proposed mechanisms for inbreeding depression – the overdominance and the partial recessive hypotheses (see Charlesworth & Charlesworth, 1999) – inbred individuals (those whose parents are related) will experience low fitness as they are relatively homozygous at loci influencing fitness. The restoration of heterozygosity by outcrossing results in an increase of fitness – heterosis (Falconer, 1989). However, it has been suggested that extreme outcrossing can disrupt favourable combinations of alleles at different loci, leading to a reduction in fitness, or outbreeding depression (Thornhill, 1993). Although the exact mechanism(s) of inbreeding depression is disputed, there is little doubt that it is a common phenomenon in numerous plant and animal species (Thornhill, 1993; Lynch & Walsh, 1998).

Most of the evidence for inbreeding depression comes from laboratory, domesticated or captive organisms (see reviews in Charlesworth & Charlesworth, 1987; Falconer, 1989; Thornhill, 1993; Lynch & Walsh, 1998), although there are some examples from the wild (e.g. Keller, 1998; Slate *et al.*, 2000a). There is some evidence that inbreeding depression is greatest in the wild (Crnokrak & Roff, 1999) and that inbreeding depression interacts with environmental heterogeneity (e.g. Dudash, 1990), so caution should be employed when drawing general conclusions from non-natural settings. However, measuring inbreeding depression in the wild is problematic for two reasons. First, in order to measure an individual's inbreeding coefficient, detailed pedigree information is required. Although molecular markers offer the opportunity to determine accurate pedigrees in natural populations, there are few populations with large numbers of individuals of known inbreeding coefficient. A second problem is that measuring fitness in the wild is

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difficult, especially in long-lived organisms (Endler, 1986). Thus, most studies of inbreeding in nature (and in the laboratory) have focused on fitness components, rather than fitness itself.

In order to measure inbreeding depression in the wild, alternative measures to the inbreeding coefficient have been used. It is known that inbreeding can generate correlations between loci throughout the genome, a phenomenon termed identity disequilibrium (Weir & Cockerham, 1973). Thus, marker heterozygosity can provide an estimate of genome-wide heterozygosity at fitness-influencing loci. For over 20 years biologists have studied correlations between heterozygosity (usually measured at allozyme loci) and components of fitness in populations of plants, insects, marine molluscs, birds, fish and mammals (for reviews see Mitton, 1993; Avise, 1994; Britten, 1996; Roff, 1997). Such approaches have the advantage that pedigree information is not required. A positive association is generally regarded as evidence that inbreeding depression or heterosis is acting on the trait in question. Typically studied traits include growth rate, seed biomass, fluctuating asymmetry or juvenile survival. Although it is likely that there is a bias in the literature, with null results remaining unreported, a recent meta-analysis concluded that positive associations between heterozygosity and fitness components are genuine and reasonably common (Britten, 1996). However, there are notable exceptions where no relationship has been detected – both in populations where inbreeding is expected to be rare (Houle, 1989; Savolainen & Hedrick, 1995) or relatively common (Whitlock, 1993).

With the advent of more variable molecular markers, alternative measures for detecting inbreeding have been proposed (e.g. Bensch *et al.*, 1994; Coulson *et al.*, 1998). Mean d^2 is a microsatellite-based measure that assumes a step-wise mutation process (Valdes *et al.*, 1993). It is assumed that the squared difference (in repeat units) between any two alleles at a locus is a linear function of the time since coalescence of the two alleles (Goldstein *et al.*, 1995). For any individual, this difference can be summed over several loci – the mean giving the measure mean d^2 (Coulson *et al.*, 1998). It is suggested (Coulson *et al.*, 1998) that mean d^2 is sensitive to both admixture between populations (relatively outbred individuals having high mean d^2 scores) and recent inbreeding (inbred individuals being homozygous at a relatively high proportion of markers and hence having low mean d^2 scores). Mean d^2 has been used to detect inbreeding depression/heterosis for fitness-related traits in wild populations of harbour seals *Phoca vitulina* (Coltman *et al.*, 1998), red deer *C. elaphus* (Coulson *et al.*, 1998, 1999; Slate *et al.*, 2000a), Arabian oryx *Oryx leucoryx* (Marshall & Spalton, 2000) and greater horseshoe bats *Rhinolophus ferrumequinum* (Rossiter *et al.*, 2001). Note that in two studies of juvenile traits in red deer (Coulson *et al.*, 1998, 1999), mean d^2 but not heterozygosity was associated with the various fitness traits, while other studies have found that mean d^2 and heterozygosity were both significant terms, acting in the same direction (Coltman *et al.*, 1998; Slate *et al.*, 2000a). Furthermore, heterozygosity but not mean d^2 was associated with female lifetime breeding success in red deer (Slate *et al.*, 2000a). Thus in some, but not all studies, mean d^2 was a more informative measure than heterozygosity. The majority of the aforementioned studies showed positive associations between fitness and the degree of outbreeding. However, Coulson *et al.* (1999) showed that relatively outbred (as measured by mean d^2) male red deer calves had reduced juvenile survival, while Marshall & Spalton (2000) found evidence for both inbreeding and outbreeding depression influencing juvenile survival in Arabian oryx.

In this paper we compare the merits of heterozygosity, mean d^2 and other related variables in detecting inbreeding depression. We also explore the number of loci and genotypes required in order to detect associations between marker-based measures and fitness. We address these issues by extending earlier studies of birth weight (Coulson *et al.*, 1998) and juvenile survival (Coulson *et al.*, 1999) in the intensively monitored, free-living population of red deer on the Isle of Rum, Scotland. Not only have inbreeding depression and/or heterosis been demonstrated in the population using marker-based measures (see above), but variation in the coefficient of inbreeding, *F*, has also been documented (Coulson *et al.*, 1998; Marshall, 1998). Thus, the study population is an appropriate one in which to investigate heterozygosity–fitness associations, as genotypic correlations between marker and trait loci are likely (Houle, 1989). The previous studies used a panel of nine microsatellite markers to detect inbreeding depression, a number typical for heterozygosity–fitness association studies. Here we use a panel of 364 animals, typed at up to 71 markers (Slate *et al.*, 2000b) enabling us to estimate genome-wide heterozygosity with a considerably larger panel of markers than any similar published study.

Methods

Study population

Data were collected in the 12 km^2 North Block of the Isle of Rum, Scotland (57°0′N, 6°20′W) where the unmanaged red deer population has been intensively monitored since 1971. Life history data collected up to 1998 were used. Culling in the study area ceased in 1973, and the population has fluctuated around carrying capacity since 1982. All animals are individually recognizable and life history data are collected from censuses performed five times per month (daily during the rutting and calving seasons). Routine genetic sampling of calves began in 1982, but almost 300 animals born prior to then were sampled by chemical immobilization or from post-mortem tissue. The animals considered in this paper

were born between 1970 and 1996. A detailed description of the study area and the first decade of the Rum red deer project can be found in Clutton-Brock *et al.* (1982).

Genetic variables

Over 1200 Rum deer have been typed at up to nine microsatellite markers (Marshall *et al.*, 1998). A subset of these animals, typed for at least seven microsatellite loci and with other appropriate life history data, were used by Coulson *et al.* (1998, 1999) in their analyses of juvenile traits. More recently, a further subset of 364 animals have been typed for up to 84 (including the original nine) microsatellite loci (Slate *et al.*, 2000b), of which 71 loci have no evidence for null alleles segregating. The subset comprises one large pedigree (the 'MAXI pedigree') descended from one very successful immigrant stag, MAXI, which was the F1 progeny of a Rum female and a mainland male (Lincoln *et al.*, 1973; Slate *et al.*, 1999). Of the 364 animals, 221 were descendants of MAXI and the remainder were 'married-ins'. The pedigree can be considered representative of the entire study population, as most calves born in recent years are descendants of MAXI. Protocols and conditions for microsatellite amplification can be found elsewhere (Marshall *et al.*, 1998; Slate *et al.*, 1998). A number of animals were not typed at all loci (mean loci per animal = 60.2, SD = 11.3) so animals typed at fewer than 20 loci were excluded from further analyses. Summing over all loci, 84.8% of genotypes were obtained. Five genetic variables were calculated from the 71 locus data set.

Multilocus heterozygosity (MLH): The proportion of typed loci for which an individual was heterozygous.

Standardized multilocus heterozygosity (sMLH)*:* The ratio of the heterozygosity of an individual to the mean heterozygosity of those loci at which the individual was typed. This measure avoids any potential bias that may be introduced by individuals being untyped at particular loci (Coltman *et al.*, 1999).

Mean d^2 (MD^2): The squared difference in repeat units between the two alleles at a locus, averaged over all loci at which an individual was scored (see Coulson *et al.*, 1998; Pemberton *et al.*, 1999).

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

Where i_a and i_b are the length in repeat units of alleles a and b at locus i and n is the number of typed loci.

Standardized mean d^2 (sM^{D2})*:* Calculated as above, but d^2 at each locus is scaled by the variance in d^2 at that locus before summing across loci. Standardizing MD^2 in this way is expected to reduce the influence of highly polymorphic loci on the overall measure (Pemberton *et al.*, 1999). In their analysis of juvenile survival Coulson *et al.* (1999) found associations between survival and MD^2, but failed to detect any association with sMD^2. However an analysis of lifetime breeding success in the study population found that the standardized measures (of MLH and MD^2) tended to explain more trait variation than their unstandardized equivalents (Slate *et al.*, 2000a).

Restricted mean d^2 (rMD^2): Five of the seventy-one loci had a distribution that included an interval of 20 base pairs or more between consecutive alleles. We regarded these distributions as potentially incompatible with a step-wise mutation process (Valdes *et al.*, 1993), and so mean d^2 was re-calculated with these loci excluded. The loci were BM1815, BM757, CSRM60, INRA11 and INRA121.

Where confusion may otherwise arise we use a subscript referring to the number of loci typed for a particular data set or replicate (e.g. sMLH$_{71}$ refers to standardized heterozygosity estimated from all 71 loci).

Traits studied

Birth weight. A continuous variable. Calves were caught and weighed in the first 2 weeks of life. Birth weight was estimated from capture weight and capture age by assuming a weight increase of 0.015 kg h^{-1} since birth (Clutton-Brock *et al.*, 1982). Although a morphometric trait, birth weight is positively associated with male lifetime breeding success (Kruuk *et al.*, 1999). In their analysis Coulson *et al.* (1998) used a data set containing 644 individuals typed at up to nine loci. In our analysis of birth weight we consider 281 individuals typed at up to 71 loci.

Juvenile survival. A binary response variable. Calf survival from 1st October in the year of birth until 1st May of the following year. Calf survival is female biased and varies from as low as 25 to almost 100% between years (Coulson *et al.*, 1999). In their analysis of juvenile survival Coulson *et al.* (1999) had sample sizes of 298 female and 275 male calves typed at up to nine loci. In this paper we considered 112 female and 103 male calves typed at up to 71 loci. Calves born prior to 1982 (when routine genetic sampling began) were excluded from analyses of juvenile survival to avoid any bias in favour of survivors.

Additional model terms

In order to improve estimates of the proportion of trait variance explained by the various genetic variables, a number of potentially confounding environmental and ecological variables were fitted to the models. Terms are listed below as either continuous or categorical factors.

Birth date. Continuous. The number of days after 1st May that a calf was born.

Density. Continuous. The number of females over 1 year old using the study area in an individual's year of birth. Density is estimated from regular censuses and an animal is regarded as resident if seen in at least 10% of censuses and in at least 4 months in a year (Clutton-Brock *et al.*, 1982).

Mean spring temperature. Continuous. The mean temperature (in °C) during April and May immediately prior to a calf's birth.

Mother's age. Continuous. Measured in years.

Mother's reproductive status. Categorical. One of five states: naïve – never previously bred, true yeld – did not breed in the previous year, summer yeld – bred in the previous year, but calf died before 1 October winter yeld – as for summer yeld, but calf died in winter, milk – successfully raised a calf in the previous year.

Sex. Categorical. Male or female.

Subdivision of study area. Categorical. The part of the study area in which an animal was usually resident. One of five areas: Shamnan Inshir, Lower Kilmory Glen, Upper Kilmory Glen, Intermediate Area and Laundry Greens.

Statistical methods

Summary statistics of the genetic variables

The correlation (Pearson's) between the different genetic variables was calculated to determine whether they were measuring similar properties. Correlations were also made between each variable when estimated from nine and 71 loci. It was assumed that 71 loci provide a reasonably accurate 'genome-wide score' for each individual, and that the correlation should indicate whether nine loci are appropriate for estimating each variable.

We calculated correlations across single loci for heterozygosity and d^2 – for example the correlation between heterozygosity at locus *a* and heterozygosity at locus *b*. These correlations were calculated for all pairs of loci and the sign and significance of each correlation noted. For both heterozygosity and d^2 we determined whether there were significantly more positive than negative correlations between loci. Although there were 2485 (71*70/2) correlations for each variable the data points were nonindependent (for example if there was a positive correlation between loci *a* and *b*, and also between *a* and *c*, then loci *b* and *c* must also be positively correlated). Thus, a simple sign test to determine whether heterozygosity and d^2 were correlated across loci would be inappropriate. We remedied this potential problem by performing a randomization-based test. Genotypes were randomized without replacement across individuals for each locus. The correlation matrix was then re-calculated for all loci, and the sign of each individual correlation noted. Thus the nonindependence between data points was retained in each replicated data set, but genotypes at each locus are randomized with respect to other loci. The statistical significance of the association between loci was calculated as the proportion of 1000 replicates for which the number of positive correlations in the matrix exceeded that obtained from the real data set. Spearman's rank correlations were performed for between locus correlations as both d^2 and heterozygosity are non-normally distributed when calculated at a single locus.

Repeating earlier analyses of inbreeding depression using a larger number of loci

Previous analyses have detected associations between MD^2 (measured at nine loci) and both birth weight (Coulson *et al.*, 1998) and juvenile survival (Coulson *et al.*, 1999). Using generalized linear modelling (GLM) techniques (McCullagh & Nelder, 1989), we tested whether these findings were robust after screening 364 animals at up to 71 loci. The significance of each of the five genetic variables was first tested in a separate model with no other terms fitted. Birth weight was tested assuming a normal error structure whereas juvenile survival was tested assuming a binomial error structure. To retain consistency with earlier studies, birth weight was modelled in a joint-sex model, whereas juvenile survival was modelled in separate-sex models. As we used five different genetic variables we performed sequential Bonferroni corrections (Rice, 1989) in order to minimize the risk of Type I error caused by multiple comparisons. Such a correction is likely to be conservative as the genetic variables are nonindependent. All analyses were implemented in SPLUS 4.5 (MathSoft).

In a second set of analyses, in addition to the genetic variables already described, a number of environmental variables were fitted into the models (see Methods, additional model terms). Initially, models were constructed with all terms fitted. Comparison was made with models in which individual terms were sequentially dropped. If the dropping of a term led to a change in model deviance that was significant at $P < 0.05$ then that term was retained in the model. Interactions between main effects were also considered. The significance of predictor terms as main effects cannot be tested if they are also included as an interaction term, and so were tested by comparison with a model containing neither the main term nor the interaction term. Categorical variables or interaction terms were not fitted if any cell sizes were lower than 30, as small cells can lead to Type I error.

Power analyses for trait-sMLH associations

A positive association between sMLH and birth weight was detected (see results), and consequently we investigated factors influencing the probability of detecting the relationship. Three factors were considered – the number of loci typed, the number of animals analysed and the heterozygosity of the loci typed.

Number of loci typed. Standardized multilocus hetrozygosity was calculated for 100 replicates of 10, 20 and 40 randomly chosen loci. Each locus was sampled without replacement from the original 71 loci. For every replicate each animal was selected for the same subset of loci. sMLH was tested in a GLM for birth weight in which the following predictor variables were included: mother's reproductive status, subdivision of study area, spring temperature and birth date. Power was determined by scoring the number of replicates (out of 100) for which sMLH explained significant variation ($P < 0.05$) in birth weight when fitted in the GLM. The sample size in all replicates was 281.

Number of individuals typed. One hundred replicates of data sets containing 25, 50, 100 or 200 randomly chosen animals were created. For each replicate a linear regression was performed, in which the relationship between $sMLH_{71}$ and residual birth weight (residuals taken from the GLM described above, minus the genetic terms) was tested. The effect of the non-genetic terms were removed prior to performing the regression because a GLM fitting the categorical terms could not have been performed for the smaller samples, causing a bias in favour of larger sample sizes. Power was determined by scoring the number of replicates for which $sMLH_{71}$ explained significant ($P < 0.05$) variation in birth weight.

Marker heterozygosity. The 71 loci were ranked in order of decreasing heterozygosity and divided into seven groups (six of ten loci and one of eleven loci). MLH and sMLH were calculated for each group, and these variables were tested as predictor terms in a GLM for birth weight ($n = 281$). The same nongenetic terms as described in the other power analyses were fitted.

The effect of marker heterozygosity on power was also tested in a second way. The correlation coefficient was calculated between the mean heterozygosity and amount of birth weight variation explained by each of the 100 ten-locus replicates of sMLH (see under 'Number of loci typed' above). A positive correlation would imply that power was greater for relatively heterozygous markers. As the distribution of the amount of trait variation explained by each replicate was positively skewed (data not shown), the significance of correlations between heterozygosity and variance explained were calculated using randomization (5000 randomizations).

All statistical tests reported are two-tailed unless indicated otherwise.

Results

Summary statistics for genetic variables

Figure 1 shows histograms of the five genetic variables for the 71-locus data set. The two MLH measures are approximately normally distributed while the MD^2 measures are all positively skewed. MD^2 calculated from all 71 loci had a bimodal distribution. Standardizing by the variance at each locus, or restricting the measure to the 66 more stepwise-compatible loci removed this bimodality, although the skew remained. Note that when MD^2 was calculated from the original nine loci used by Coulson *et al.* (1998, 1999) the distribution was not bimodal. Clearly the bimodality arises from the inclusion of the less stepwise loci. As with previous analyses in this population (Coulson *et al.*, 1998, 1999) we did not log transform any of the MD^2 derived measures, although this measure has been transformed elsewhere (Coltman *et al.*, 1998).

The correlation coefficients between the five genetic variables when calculated from 71 loci are given in Table 1. Predictably, the two MLH measures were very highly positively correlated to each other, and both were significantly positively correlated to all of the MD^2 derived measures. sMD^2 was negatively (but not significantly) correlated to the other MD^2 measures, while only MD^2 and rMD^2 were positively and significantly correlated to each other.

The correlations between genetic variables estimated from the original nine loci and from up to 71 loci are given in Table 1. When calculated from 71 loci both MLH measures were positively and significantly correlated with their nine locus equivalents. sMD^2 approached significance when the correlation between the nine locus and 71 locus estimates was calculated ($r = 0.100$, $P = 0.068$). However, nine locus MD^2 was not significantly correlated to 71 locus MD^2 ($r = 0.047$, $P = 0.396$). A comparison was also made between each of the nine locus variables when calculated from the overall population ($n = 1105$) or only those animals in the MAXI pedigree ($n = 346$). For each variable both the mean and variance were almost identical for the two groups of animals (data not shown) suggesting that the MAXI pedigree is representative of the overall population with respect to these genetic variables.

In their analysis of juvenile survival Coulson *et al.* (1999) measured individual d^2 scores at each of the nine typed loci and then examined correlations across loci. A significant proportion (25/36, $P < 0.05$) of the correlations were positive. We extended this analysis to 71 loci and also investigated correlations between heterozygosity at each locus using randomization approaches to test significance (see methods). When heterozygosity at each locus was examined there were significantly more positive correlations than expected by chance (1294

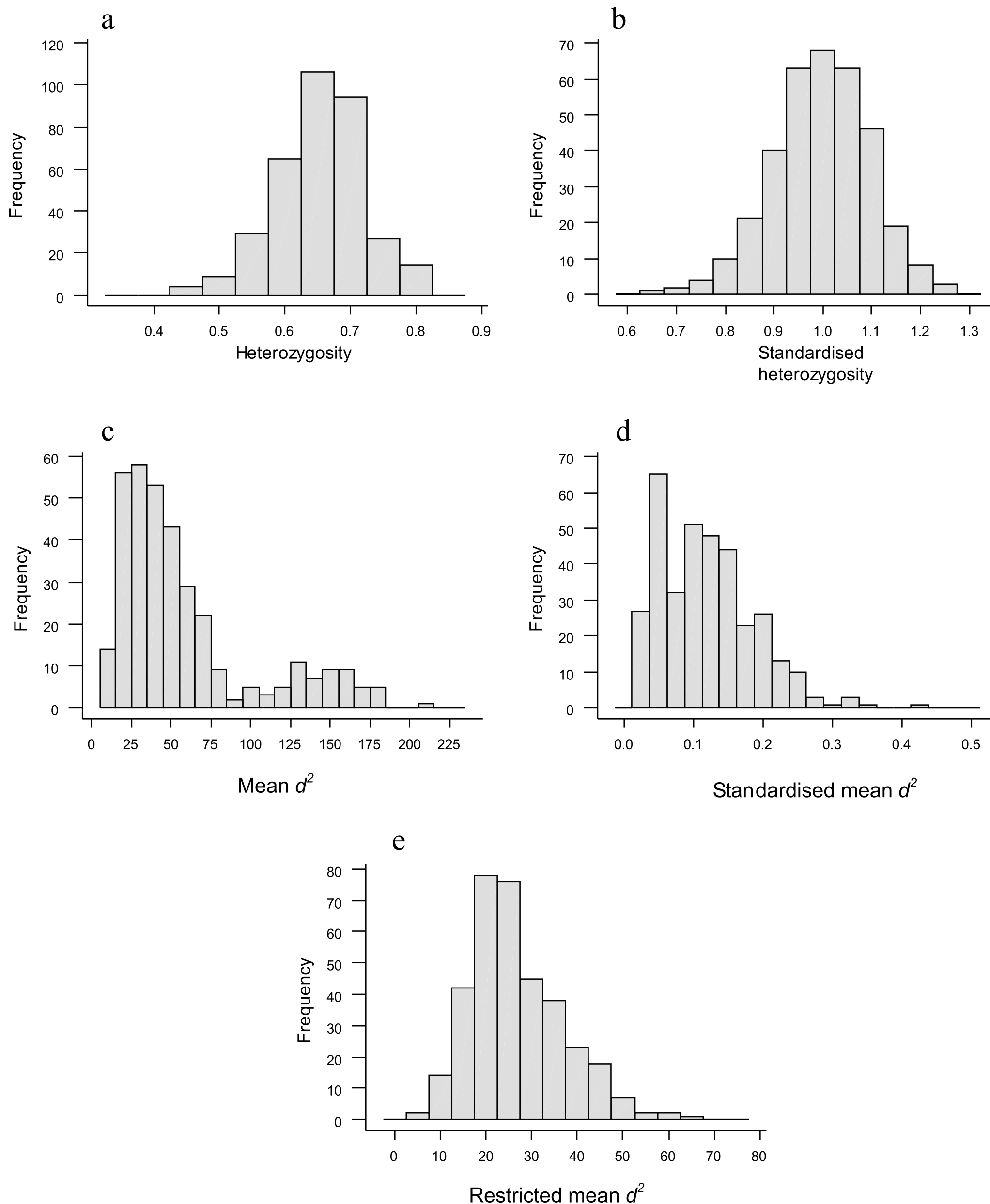

Fig. 1 Histograms of (a) MLH (b) sMLH (c) mean d^2, (d) standardized mean d^2 and (e) restricted mean d^2 for the 346 animals typed at up to 71 loci.

positive vs. 1191 negative, $P = 0.043$), but there were no more significant (at $P < 0.05$) correlations than expected by chance (146 significant correlations, binomial test $P = 0.053$). Amongst the significant correlations more were positive than negative (88 positive vs. 58 negative, sign test, $P = 0.016$). Thus heterozygosity is positively, but weakly, correlated across loci, indicating that MLH can distinguish between relatively inbred and outbred animals in the population.

Unlike heterozygosity, individual d^2 scores were not positively correlated between loci more often than expected by chance (1246 positive vs. 1239 negative correlations, $P = 0.91$). More correlations were significant (at $P < 0.05$) than expected by chance (170 significant, binomial test, $P < 0.001$), but amongst this subset there was no evidence that individual d^2 scores were positively correlated across loci (92 positive vs. 78 negative, sign test, $P = 0.32$).

Table 1 Correlation coefficients between (a) the five genetic variables estimated from 346 animals typed at up to 71 loci (b) 9 and 71 locus estimates of each genetic variable. In (a) the above diagonals represent the correlation coefficient (*r*) and the below diagonals represent the significance of the correlation. Note that MD2 and sMD^2 are negatively, but nonsignificantly correlated when typed at up to 71 loci. As all of the original nine loci were compatible with the stepwise mutation model, rMD2 is not shown in (b).

Variable	MLH	sMLH	MD^2	sMD^2	rMD^2
(a)					
MLH	–	0.984	0.145	0.250	0.251
sMLH	<0.001	–	0.152	0.275	0.254
MD^2	0.007	0.005	–	–0.054	0.186
sMD^2	<0.001	<0.001	0.321	–	–0.007
rMD^2	<0.001	<0.001	<0.001	0.894	–
	r	*P*			
(b)					
MLH	0.313	<0.001			
sMLH	0.323	<0.001			
MD^2	0.047	0.396			
sMD^2	0.100	0.068			

Associations between juvenile traits and genetic variables scored at 71 Loci

Birth weight

When tested alone in a joint sex model ($n = 281$), both MLH71 ($F_{1,280} = 9.64$, $P = 0.002$) and $sMLH_{71}$ ($F_{1,280} = 8.78$, $P = 0.003$) explained significant variation in birth weight (with relatively outbred animals being born heaviest). Both variables remained significant after sequential Bonferroni correction. In contrast none of the three MD^2 based measures were significant terms ($0.334 < P < 0.411$). A full model with the following nongenetic terms was also constructed: subdivision of study area, mother's reproductive status, spring temperature, and birth date. The sex of the calf was not a significant term in this model and the interaction terms between sex and any genetic term were also not significant. The full model (with sMLH fitted) is shown in Table 2. MLH explained a similar amount of variation in birth weight to sMLH and was also a significant term. Again, both terms remained significant after Bonferroni correction. None of the three MD^2 derived variables explained significant variation in birth weight, nor did they approach significance ($0.860 < P < 0.947$). Thus, as in the earlier analysis of Coulson *et al.* (1998), relatively outbred animals were born heavier than their more inbred counterparts. However, in contrast to the earlier study we detected the inbreeding/birth weight association using MLH-derived measures rather than MD^2-derived measures.

We also constructed models in which heterozygosity at each typed locus was fitted, in an attempt to identify particular regions of the genome that might be associated with variation in birth weight. Animals were coded as 0 if homozygous and 1 if heterozygous at any locus. Locus-specific heterozygosity was fitted as a two-level categorical variable in a GLM containing the

Table 2 (a) GLM for birth weight for animals typed at up to 71 loci. Null model deviance = 387.8; full model deviance = 288.9; deviance explained = 25.5%; $n = 281$. F refers to the change in deviance explained by the model when that term is dropped. All significant terms are shown, in order of decreasing deviance explained. Deviance explained refers to the absolute amount and the percentage of null model deviance explained by each term. sMLH remains significant after sequential Bonferroni correction. (b) GLM for birth weight with locus-specific and multilocus terms fitted. Significant genetic terms include $sMLH_{71}$ and heterozygosity at two unlinked microsatellite markers (BM2934 and SPS113). $sMLH_{71}$ remains significant after correction for multiple testing. Null model deviance = 347.6; full model deviance = 229.9; deviance explained = 33.9%; $n = 244$.

Term	Term type	d.f	*F*	*P*	Direction of association	Deviance explained
(a)						
Mother's reproductive status	Factor (5 levels)	4,273	10.24	<0.0001	Naïve lightest, yeld heaviest	44.0 (11.3%)
Area	Factor (5 levels)	4,273	4.45	<0.005	Laundry Greens heaviest, Lower Kilmory Glen lightest	19.1 (4.9%)
Birth date	Continuous	1,270	13.71	<0.001	+ve	14.7 (3.8%)
Mean spring temp.	Continuous	1,270	12.85	<0.001	+ve	13.8 (3.6%)
sMLH	Continuous	1,270	7.77	<0.005	+ve	8.4 (2.2%)
(b)						
Mother's reproductive status	Factor (5 levels)	4,234	11.50	<0.0001	Naïve lightest, yeld heaviest	46.0 (13.3%)
Area	Factor (5 levels)	4,234	5.11	<0.001	Lower Kilmory Glen lightest, Laundry Greens heaviest	20.4 (5.9%)
Birth date	Continuous	1,231	13.80	<0.0005	+ve	13.8 (4.0%)
Mean spring temperature	Continuous	1,231	10.41	<0.005	+ve	10.4 (3.0%)
H_{SPS113}	Factor (2 levels)	1,231	9.72	<0.005	Heterozygotes heaviest	9.7 (2.8%)
H_{BM2934}	Factor (2 levels)	1,231	6.84	<0.01	Heterozygotes heaviest	6.8 (2.0%)
$sMLH_{71}$	Continuous	1,231	6.84	<0.01	+ve	6.8 (2.0%)

non-genetic terms listed in Table 2. Heterozygotes were significantly heavier than homozygotes at two unlinked loci, SPS113 and BM2934. As a large-number of loci (71) were tested, problems of multiple comparisons arise and the power to detect significant single-locus effects is compromised by a conservative Bonferroni correction. In fact, neither result remained significant after Bonferroni correction, although we note that both loci appear to be linked to quantitative trait loci (QTL) for birth weight identified in a mapping study (Slate, 1999). In order to determine whether SPS113 and BM2934 were contributing a disproportionate amount of the effect due to $sMLH_{71}$, we constructed a model containing all three terms (see Table 2). sMLH71 remained a significant term suggesting that genome-wide heterozygosity was correlated with birth weight. Although only two loci showed significant associations with birth weight, heterozygotes were heavier than homozygotes at significantly more than half of the 71 loci (heterozygotes heaviest at 45 loci vs. homozygotes heaviest at 26 loci; sign test, $P = 0.032$).

Juvenile survival

When fitted alone no genetic terms explained significant variation ($0.292 < P < 0.869$) in male calf survival ($n = 103$). In more detailed models, in which density ($P < 0.05$), birth weight ($P < 0.005$) and an interaction between spring temperature and density ($P < 0.05$) were significant terms, none of the genetic terms approached significance when fitted as main or interaction terms. Thus, we found, no support for the earlier conclusion that relatively inbred male calves had enhanced juvenile survival Coulson *et al.* (1999).

In models of female calf survival ($n = 112$) none of the genetic variables were associated with first winter survival when fitted as the only term ($0.088 < P < 0.700$). In a full model retaining the terms spring temperature ($P < 0.05$) and an interaction between density and spring temperature ($P < 0.001$), none of the genetic variables were significant when fitted as main terms ($0.341 < P < 0.485$). Thus, the 71 locus data set provides no evidence to support Coulson *et al.*'s (1999) observation that relatively outbred female calves had enhanced first winter survival.

Power to detect the association between birth weight and sMLH

Number of loci typed

The probability of detecting the birth weight–sMLH association increased with the number of loci typed (Table 3). When only 10 loci were used to calculate sMLH, power was low (18/100 replicates). We also calculated the mean correlation between sMLH when calculated at either 10, 20 or 40 loci with the 71-locus estimate of sMLH. As expected, the correlation coefficient increased with the number of loci typed (Table 3).

Table 3 (a)The influence of the number of loci typed on power to detect the sMLH–birth weight association. Power was calculated as the number of replicates (out of 100) for which sMLH explained significant variation ($P < 0.05$) in birth weight. The mean correlation between $sMLH_{71}$ and $sMLH_x$, where × is either 10, 20 or 40 loci is also shown. $n = 281$ for each replicate. (b) The influence of number of individuals typed on power to detect the $sMLH_{71}$–birth weight association. Power was calculated by performing a linear regression of $sMLH_{71}$ on residual birth weight (using residuals from the birth weight GLM with genetic terms excluded). Note that the *F* ratio required for significance (at $P < 0.05$) decreases with increasing sample size. The mean *F* ratio obtained, and hence power, increases with increasing sample size.

Number of loci	Power	Mean (SD) correlation with $sMLH_{71}$	
(a)			
10	0.18	0.425 (0.053)	
20	0.31	0.572 (0.055)	
40	0.63	0.784 (0.042)	
Sample Size	Required *F*	Mean *F* (SD)	Power
(b)			
25	4.28	1.77 (2.85)	0.12
50	4.04	2.48 (2.54)	0.21
100	3.93	3.85 (3.00)	0.42
200	3.88	5.43 (2.00)	0.71

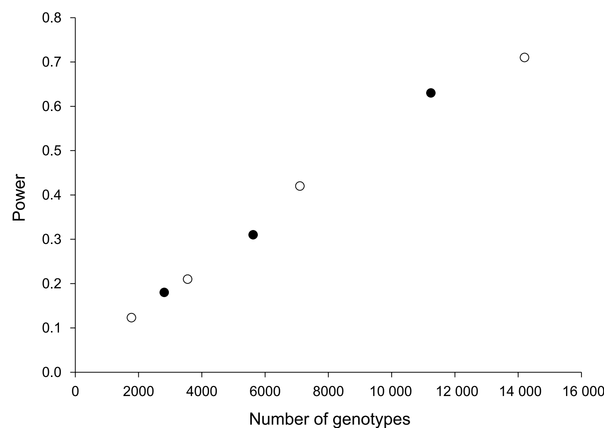

Fig. 2 Effect of the number of genotypes on power. Filled circles represent power calculations where the number of loci was varied, and open circles represent power calculations where the number of individuals typed was varied. There is no apparent advantage to typing additional loci at the expense of fewer individuals or *vice versa*. The relationship is linear for at least the first 10 000 genotypes.

Number of individuals typed

The probability of detecting the sMLH–birth weight association was dependent on sample size (Table 3). However with only 25 individuals sampled, the association was still detected for 12 of 100 replicates (at $P < 0.05$).

Plotting the number of genotypes scored against power (Fig. 2) suggests that the number of loci scored and

Table 4. The influence of locus variability on power to detect the sMLH–birth weight association.

Locus group	Mean (SD) heterozygosity of loci in group	Within group variance in individual		Birth weight variation explained by sMLH		Birth weight ariation explained by MLH	
		sMLH	MLH	F	P	F	P
1	0.868 (0.015)	0.022	0.017	0.46	0.50	0.45	0.50
2	0.812 (0.021)	0.026	0.017	0.82	0.37	0.84	0.36
3	0.766 (0.016)	0.046	0.027	4.61	**<0.05**	4.60	**<0.05**
4	0.710 (0.017)	0.057	0.029	0.58	0.45	0.58	0.45
5	0.666 (0.011)	0.072	0.032	1.35	0.25	1.42	0.23
6	0.572 (0.089)	0.084	0.028	1.56	0.21	1.42	0.24
7	0.233 (0.090)	0.404	0.021	1.00	0.32	0.80	0.37

Loci were ranked in order of decreasing heterozygosity and then sorted into groups of 10 (except the lowest heterozygosity group which contained 11 loci). The mean heterozygosity and SD of the 10 loci in each group is shown in the second column. MLH and sMLH were fitted in the birth weight GLM described in Table 2. There was no evidence that power was associated with marker heterozygosity or within-group variance in marker heterozygosity. Only group 3 (bold) explained significant variance in birth weight. Note that this group included the locus SPS113.

sample size are equally important in determining power to detect sMLH–trait associations.

Variability of loci typed

When MLH and sMLH were calculated from seven groups of ten loci (ranked in order of decreasing heterozygosity), the relationship with birth weight was detected by only one of the seven groups (the third most variable, see Table 4). There was no evidence that more variable groups of markers were better at detecting an association between MLH and birth weight. We also calculated the between-individual variance in MLH and sMLH for each of the seven groups. There was no evidence that sets of markers with high within-group variance in MLH and sMLH were better at detecting the association between birth weight and MLH (Table 4). It is plausible that marker heterozygosity has a weak influence on power, but any effect is masked by the fact that the power to detect associations is limited when only ten loci are used (see above).

There was no significant relationship between a 10-locus replicate's mean heterozygosity and the amount of deviance it explained in birth weight ($r = -0.093$, $P = 0.36$). When replicates containing the loci SPS113 or BM2934 (which may be linked to loci influencing birth weight; see above) were removed the correlations remained nonsignificant (number of replicates = 76; correlation coefficients not shown). Thus, this analysis found no evidence for a positive relationship between marker heterozygosity and power.

Discussion

Are earlier studies supported by the larger panel of 71 Loci?

The earlier studies of inbreeding depression for juvenile traits in this population used the genetic variables MLH and MD^2 calculated from nine loci (Coulson *et al.*, 1998, 1999). Using a larger panel of 71 loci we found some support for the earlier findings. In concordance with Coulson *et al.* (1998) we found that relatively outbred calves were born heavier than their more inbred counterparts. However, in their analysis of juvenile survival Coulson *et al.* (1999) concluded that relatively outbred females had a greater probability of survival, whereas relatively outbred male calves had *decreased* survival to age 1 year. Using the panel of 71 markers we found little evidence for either relationship and none of the genetic terms were significant when fitted as main or interaction terms in juvenile survival models.

There are a number of possible reasons for discrepancies between this and earlier studies. First, many of the animals used in the 71-locus analysis were descended from a recently introduced stag, MAXI's father (see methods; Slate *et al.*, 1999). Although there was no discernible difference between the MAXI pedigree and the overall population in terms of the genetic variables used in the analyses, it is nonetheless possible that the genetic architecture of traits differs between the two groups of animals. Indeed, a GLM of birth weight, that accounted for potentially confounding environmental factors revealed MAXI descendants to be significantly heavier than non-descendants (Slate, 1999). However, both this data set and the one used by Coulson *et al.* (1999) included both MAXI descendants and 'married-ins', so the influence of different genetic architectures is likely to be small. Secondly, in their analyses of juvenile survival Coulson *et al.* (1999) used a larger sample size ($n = 573$) than this study ($n = 215$), although the number of genotypes used in this analysis was approximately three times greater than in the earlier analysis. However, even with three times the number of genotypes the power to detect an association (with $r = 0.183$) between MLH and juvenile survival is only about 0.5 (inferred from Fig. 2). Alternatively, the previously detected

associations between MD^2 and juvenile survival could be attributable to Type I error, although they were robust to bootstrapping in the original analysis (Coulson *et al.*, 1999).

How informative are MLH- and MD^2-based measures?

It was striking that all of the significant associations detected in this study were with MLH or sMLH whereas the earlier studies only detected associations with MD^2 (Coulson *et al.*, 1998, 1999). This study raises some doubt over the efficacy of MD^2 to detect inbreeding depression or heterosis. There was no evidence that within-individual d^2 scores were correlated across loci. Thus, a very large number of loci may be required to accurately reflect mean coalescence times between parental alleles. Furthermore, the degree to which average coalescence times measure ancestral inbreeding is unknown. In contrast heterozygosity was correlated across loci, albeit weakly. When measured at 71 loci, the correlation coefficient between MLH and MD^2 ($r = 0.145$) was lower than that reported in previous studies using fewer loci. For example, Coltman *et al.* (1998) reported a correlation coefficient of 0.30 in a population of harbour seals (*P. vitulina*), while the correlation between the two variables in the studies by Coulson *et al.* (1998, 1999) was 0.38 (see Pemberton *et al.*, 1999). Thus, it appears that MD^2 is less closely related to genome-wide heterozygosity than previous studies would suggest.

A potential difficulty with MD^2 may arise when certain loci contribute disproportionately to the overall score. In an attempt to alleviate this problem we used several approaches. First we recalculated the statistic with five loci removed (rMD^2). Secondly we calculated the statistic but scaled the score at each locus by the variance in d^2 at that locus, before summing over all loci (sMD^2). We found no association between any trait and either MD^2 related variable. However Slate *et al.* (2000a) did find that sMD^2 had a tendency to explain more variation in adult lifetime breeding success and to be more consistent with results obtained using sMLH than did unstandardized MD^2. Problematically, there is no obvious way in which to distinguish between conflicting causes of high variance in locus-specific d^2 measures. For example, a large variance could represent information about coalescence times between the two parental alleles, or could be an artefact of either a nonstepwise mutation process or a high mutation rate. Such conflicting processes make it difficult to know whether or not to scale MD^2, and whether or not loci with high variance in d^2 should be included. Furthermore, evidence is accumulating that microsatellites do not evolve in a simple stepwise process (Ellegren, 2000), suggesting that the rationale behind the use of MD^2 may be flawed.

We regard MLH and sMLH as more robust measures with which to detect inbreeding depression/heterosis and to measure dominance variance than any of the MD^2-based measures. A recent simulation study also suggests that MLH is preferable to MD^2 under a range of mutation model and population history scenarios (Tsitrone *et al.* In Press). In broad agreement with these findings, a recent study of grey wolves (*Canis lupis*) found that MLH outperformed MD^2 as a predictor of inbreeding coefficient (Hedrick *et al.*, 2001), in a recently inbred, captive population. Gaps in the pedigree meant that we were unable to calculate individual inbreeding coefficients in our study population, making a similar analysis impossible.

Power to detect sMLH–trait associations

While correlations between heterozygosity and fitness have been reported for many organisms and traits, there are notable exceptions for which no association was found. For example, Whitlock (1993) failed to find any association between MLH (measured at five allozyme loci) and a number of traits in the forked fungus beetle (*Bolitotherus cornutus*), despite a large sample size of over 900. Furthermore, it is almost certain that there is a bias in the literature, so that studies that fail to show any association remain unreported. A meta-analysis of plants and animals by Britten (1996) suggested that the mean correlation coefficient between MLH and growth rates was weak yet positive (mean $r = 0.181$, 95% CI = 0.158–0.207). We detected an almost identical ($r = 0.183$) correlation between $sMLH_{71}$ and birth weight, yet the power to detect this association was only 0.18 when ten loci were used (Table 3). Most MLH-fitness trait studies to date have typically used between five and ten loci. Hence it is unsurprising that some studies fail to detect sMLH–trait associations.

Although attention has been given to the fact that large sample sizes are required to confirm the absence of any association (i.e. to avoid Type II error), the effect of the number of loci typed has been largely ignored. The power analysis presented here suggests that sample size and number of typed loci are equally important in detecting associations. Thus, the number of scored genotypes should be taken into consideration before rejecting the possibility of Type II error. For example, although Whitlock (1993) screened 900 beetles, only five loci were scored, giving a total of under 5000 genotypes. Extrapolating from Fig. 2, the power to detect a significant (at $P < 0.05$) MLH–trait association with correlation coefficient of 0.18, was only about 0.25 for that study.

Some caution should be taken when interpreting the power analyses, as there was a degree of nonindependence between each replicate (as some replicates had loci in common). In particular, replicates containing SPS113 or BM2934 were often significantly associated with birth weight. The most probable consequence of any such nonindependence is that power was slightly over-estimated. If this nonindependence is taken into account, then an even greater sample size is required in order to reject Type II error.

Causal mechanisms of the MLH–birth weight association

Three genetic mechanisms have been proposed to explain fitness–heterozygosity associations (for reviews see Savolainen & Hedrick, 1995; David, 1998; Lynch & Walsh, 1998). The first hypothesis, termed *true overdominance*, relies on the fact that the marker loci themselves are functional, and assumes that heterozygous genotypes have a greater fitness than homozygotes at the typed markers. Clearly, this mechanism does not apply to microsatellite loci, which are predominantly found in non-coding regions of the genome. A second hypothesis, *associative overdominance*, relies on the markers being in linkage disequilibrium with loci affecting the trait. Note that the associative overdominance mechanism can work if the linked loci are overdominant or have segregating partially recessive alleles (Lynch & Walsh, 1998). The third hypothesis assumes that heterozygosity at marker loci reflects genome-wide levels of heterozygosity. Associations between unlinked loci have been termed *identity disequilibrium* (Weir & Cockerham, 1973) and are generated by inbreeding in the population. Positive correlations between heterozygosity and fitness are attributable to inbred individuals being relatively homozygous at both marker and trait loci. Note that the first two mechanisms involve effects local to the typed markers, whereas the third mechanism involves genome-wide genetic variation. Two notable studies of randomly mating populations – one in *Drosophila* (Houle, 1989) and one in Scots pine (Savolainen & Hedrick, 1995) – have failed to find significant associations between heterozygosity and fitness. In panmictic populations heterozygosity–fitness associations can only be generated by one of the first two mechanisms. Given the lack of association in both studies the authors concluded that the third hypothesis was the most likely cause of heterozygosity–fitness associations. The Rum red deer population, with its documented cases of inbreeding offers limited scope for differentiating between the three mechanisms. However, the fact that we detected the relationship between birth weight and heterozygosity with multiple sets of ten loci provides some support for a general rather than a local genetic cause of our association. Thus, our data support the conclusions of Houle (1989) and Savolainen & Hedrick, 1995).

Conclusions

Using a panel of 71 microsatellite loci we confirmed a previous observation that relatively outbred red deer calves were born heavier than their more inbred counterparts. However we found no support for earlier studies of juvenile survival. In detecting these associations heterozygosity related variables performed better than the recently derived measure MD^2. It is unclear how reliable MD^2 based measures are, and will probably remain so until the microsatellite mutation process is better understood. Indeed, recent simulations show that MD^2 performs less well than MLH under a range of mutation scenarios (Tsitrone *et al.* In Press). It is possible that one or some loci with high mutation rates or non-stepwise mutation events generate enough 'noise' to swamp genome-wide inferences made from other loci. The greater the number of loci typed, then the greater is the probability that a 'noisy' locus will be encountered. We conclude that sMLH is a more useful variable for measuring inbreeding depression and dominance variance than MD^2-based variables. Furthermore, power is likely to remain low unless large numbers of genotypes ($n > 10\,000$) are generated, or the study trait has particularly high levels of dominance variance. Finally, although molecular measures are clearly useful as tools to detect inbreeding depression, it is evident from this study that they will not detect all cases of inbreeding depression, and cannot be used to reject its existence.

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