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Microsatellite Diversity and Fitness in Stranded Juvenile Harp Seals (*Phoca groenlandica*)

Maria Kretzmann, Laura Mentzer, Robert DiGiovanni Jr., Matthew S. Leslie, and George Amato

From the Southampton College of Long Island University, 239 Montauk Highway, Southampton, NY 11968 (Kretzmann and Mentzer); the Riverhead Foundation for Marine Research and Preservation, 428 East Main Street, Riverhead, NY 11901 (DiGiovanni); and the American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024 (Leslie and Amato). Maria Kretzmann is now at 1737 Hill Drive, South Pasadena, CA 91030.

Address correspondence to Dr. M. Kretzmann at the address above, or e-mail: maria.kretzmann@liu.edu.

Abstract

A positive relationship between genetic diversity at neutral markers and juvenile survival has been demonstrated for many vertebrate populations, although the correlation is typically weak and the explanation for it remains controversial. We assessed variation at 9–12 microsatellite loci in 65 juvenile harp seals (*Phoca groenlandica*) that stranded in poor condition around Long Island, NY, from 2001 to 2004. Compared with seals that died, surviving individuals had slightly higher measures of mean d^2, which reflects the size difference between alleles within an individual and provides an index of outbreeding. In contrast, there were no significant differences between survivors and nonsurvivors in heterozygosity or estimates of internal relatedness. This pattern is attributed to the fact that these microsatellite markers were exceptionally variable in this species (9–22 alleles per locus), and all individuals were heterozygous at most loci. Under these circumstances, mean d^2 may provide a powerful measure for assessing diversity–fitness correlations.

The relationship between genetic variation and fitness has long been of interest to evolutionary and conservation biologists. Relatively weak heterozygosity–fitness correlations have now been documented in many species, using several types of genetic markers and various estimates of diversity (Britten 1996; Mitton 1997). Microsatellite markers are the most commonly employed neutral markers in studies of population-level genetic variability. In addition to heterozygosity estimates based on microsatellites, some authors have used another measure of variation known as mean d^2 (Coulson et al. 1998). Mean d^2 measures allele size differences within an individual, which should provide an index of outbreeding, based on the stepwise model for microsatellite mutation (Valdès et al. 1993). Significant correlations between mean d^2 estimates and juvenile survival have been reported for red deer (*Cervus elaphus*; Coulson et al. 1998), harbor seals (*Phoca vitulina*; Coltman et al. 1998), and greater horseshoe bats (*Rhinolophus ferrumequinum*; Rossiter et al. 2001). In all these studies, mean d^2 was more strongly correlated with survival than was individual heterozygosity.

There is also evidence that the other major component of evolutionary fitness, reproductive success, is correlated with microsatellite diversity estimates. Höglund et al. (2002) showed that mean d^2 was significantly positively correlated with lifetime reproductive success for male black grouse (*Tetrao tetrix*), whereas the relationship between individual heterozygosity and fitness was not quite significant. Amos et al. (2001) examined data sets for 3 species (gray seal *Halichoerus grypus*, long-finned pilot whale *Globicephala melas*, and wandering albatross *Diomedea exulans*). They found significant correlations between lifetime success and standardized heterozygosity estimates, whereas mean d^2 was more weakly correlated with fitness. These authors also introduced a new measure of microsatellite diversity known as internal relatedness (IR), which weights homozygotes for rare alleles more heavily (as being more likely derived from related parents) than homozygotes for common alleles (Amos et al. 2001). This measure was also significantly (negatively) correlated with reproductive success in their data sets, as well as others (Hoffman et al. 2004; Seddon et al. 2004).

Mean d^2 estimates of parental relatedness are thought to reflect events deeper in the pedigree than individual heterozygosity, such as mixing of genetically divergent populations (Coulson et al. 1998), and may therefore be more appropriate for detecting outbreeding rather than close inbreeding (Neff 2004). Indeed, mean d^2 was shown to be a poor predictor of

both inbreeding and fitness in a captive wolf (*Canis lupus*) population (Hedrick et al. 2001), as well as Darwin's ground finch species *Geospiza fortis* and *Geospiza scandens* (Markert et al. 2004). A recent meta-analysis of microsatellite studies concluded that multilocus heterozygosity was usually a better index of inbreeding than was d^2 and that associations between fitness and genetic variation are generally weak (Coltman and Slate 2003). Theoretical work has also led to the conclusion that fitness should be more closely correlated with heterozygosity than with mean d^2 under most scenarios, unless the product of marker mutation rate and effective population size is substantially greater than one (Tsitrone et al. 2001). For microsatellite markers with mutation rates of 10^{-4} to 10^{-3}, d^2 should therefore outperform heterozygosity as an index of fitness only if population size is on the order of 1000–10 000 individuals (Goudet and Keller 2002).

Harp seals (*Phoca groenlandica* or *Pagophilus groenlandicus*) are among the most numerous pinnipeds in the world and breed at 4 main locations in the Arctic: the Gulf and Front populations near Newfoundland, Canada, as well as Greenland Sea and White Sea populations in the northeastern Atlantic (Bowen and Siniff 1999). Annual pup production in the Canadian Arctic alone was recently estimated at approximately 1 million (Stenson et al. 2003). The species has apparently extended its traditional range southward since the mid-1990s, with increasing numbers observed in Nova Scotia and northern New England (McAlpine and Walker 1990; McAlpine et al. 1999; Harris et al. 2002; Lucas and Daoust 2002). Around Long Island, NY, the Riverhead Foundation for Marine Research and Preservation has documented a substantial increase in stranded juvenile harp seals, which first appeared in the area in 1993 and reached a peak of more than 100 individuals in 2001 (DiGiovanni R, unpublished data).

Little is known of harp seal genetic diversity and population structure. Meisfjord and Sundt (1996) reported that both allozyme and multilocus DNA fingerprinting analyses revealed differences between harp seals in the northeastern and northwestern Atlantic, but not between the White and Greenland Sea populations. Similarly, sequence data from the mitochondrial DNA cytochrome *b* gene supported genetic differences only between Canadian populations and the northeastern Atlantic breeding groups (Perry et al. 2000). DNA fingerprint band-sharing estimates were low, both within and between populations (Meisfjord and Sundt 1996), indicating high genetic diversity, as expected for a species with very large population sizes. It is possible that finer scale genetic differences may be revealed when all breeding populations can be assessed with more variable genetic markers, such as microsatellites.

The goal of this study was to use microsatellite markers to examine patterns of genetic diversity in harp seals stranding around Long Island, NY. All stranded individuals were found in poor condition, but some recovered and were released, whereas others did not survive. Because all seals in the stranding program were protected from other sources of mortality (i.e., starvation, predation, and accidental death), we hypothesized that survivors have some innate fitness advantage that would be reflected in increased genetic variability. We also wanted to evaluate the relative effectiveness of the microsatellite measure mean d^2 as an index of fitness in a species in which most individuals are expected to be heterozygous at most marker loci. In this case, mean d^2 may be expected to perform better than heterozygosity or IR measures.

Methods

Samples were obtained from 65 juvenile harp seals that stranded in poor condition around Long Island, NY, and were recovered by the Riverhead Foundation for Marine Research and Preservation (Riverhead, NY) between 2001 and 2004 (see Supplementary Material). Twenty-eight of these seals did not survive; 6 were found dead, 4 others died in transit to the rehabilitation facility, 8 were euthanized (up to 59 days later), and the others died 1–24 days after rescue (see Supplementary Material). Necropsies were performed on these individuals and muscle samples obtained for DNA analysis. Seven individuals had congested lungs, 6 had rocks and/or other debris in the stomach, 2 had heavy parasite loads, 2 had infected jaws, 2 had kidney failure/high urea levels, 1 failed to recover from a seizure, and 1 had injuries consistent with a boat propeller strike. There were no definitive findings in the remaining 7 necropsies. The 37 seals that recovered after rehabilitation were released after 16–155 days. These individuals initially suffered from most of the same ailments as the nonsurvivors, that is, respiratory congestion, skin lesions, sand and/or rocks in stomach, and/or malnourishment (see Supplementary Material). Surviving seals were sampled using a leather punch to remove tissue from the rear flipper webbing, as part of the prerelease tagging procedure. All samples were frozen or preserved in salt-saturated dimethyl sulfoxide (Amos and Hoelzel 1991) prior to analysis.

DNA was extracted from tissue samples by digestion overnight in 750 μl of lysis buffer (0.1 M Tris, 0.1 M ethylenediaminetetraacetic acid [EDTA], 0.25 M NaCl, 0.5% Triton, 2% sodium dodecyl sulfate, and 0.5 mg/ml proteinase K) at 50° C, followed by phenol/chloroform/isoamyl alcohol extractions (Sambrook et al. 1989), and precipitation in 100% cold ethanol overnight. Pellets were rinsed twice with 70% ethanol, air-dried, resuspended in Tris–EDTA and refrigerated or frozen until used. We used 12 microsatellite primer pairs, isolated in several different pinniped species and known to amplify polymorphic loci in harp seals (Coltman et al. 1996; Gemmell et al. 1997; Davis et al. 2002). Each forward primer was labeled with TET, HEX, or FAM fluorescent dye (Integrated DNA Technologies, Coralville, IA). We followed polymerase chain reaction (PCR) protocols given in Gemmell et al. (1997), except for primers Hg6.1 and Hg6.3, which required more stringent annealing temperatures (54–58° C).

PCR products were visualized under UV on agarose gels containing ethidium bromide and diluted for genotyping according to amplification strength. Diluted samples (1 μl) were mixed with 10 μl of 97.6% formamide and 2.4% ROX-500 size standard (Applied Biosystems) and denatured at 94° C for 3 min before loading in an Applied Biosystems

(3700 or 3730XL) automated sequencer. Multiple loci with nonoverlapping allele sizes and/or different dyes were run in the same capillary when possible. Allele size data were collected using GENESCAN and GENEMAPPER software packages (Applied Biosystems). Several samples of known genotype were used to standardize slight (1–2 bp) allele size differences between instruments. Statistical analysis for Hardy–Weinberg (HW) equilibrium, linkage disequilibrium, and allele frequency differences between survivors and nonsurvivors was performed with GENEPOP (Web version 3.4, probability tests using the Markov chain method; Raymond and Rousset 1995). The *t*-tests for differences in mean d^2 between survivors and nonsurvivors were performed using MICROSOFT EXCEL. Although these data are not normally distributed, the parametric *t*-test is robust to violations of normality. The nonparametric equivalent Mann–Whitney test did not provide more power to resolve differences in this case (data not shown).

Results

A total of 65 harp seal juveniles (28 nonsurvivors and 37 survivors) were genotyped at 9–12 dinucleotide microsatellite loci (see Supplementary Material). Locus *Pv16* was least variable with 9 alleles detected in 61 individuals, whereas locus *Hl15* was unusually variable with 22 alleles in 60 seals. Nearly all the possible allele sizes between largest and smallest were detected at each locus, many in single individuals. Across all loci, the same alleles were generally common in both groups of seals, and allele frequency differences between survivors and nonsurvivors were not significant overall ($\chi^2 = 35.16$, degrees of freedom [df] = 24, $P = 0.07$). Two individual loci (*Hg4.2* and *Hg6.1*) did show significant allele frequency differences between these 2 groups of seals ($P = 0.05$ and 0.01). For 3 loci (*Hg6.1*, *Hg8.10*, and *Hl15*), the nonsurvivors had 1 or 2 very common alleles, whereas the survivors did not (see Supplementary Material). More rare alleles were detected among the survivors in many cases, which is not surprising given that we had samples from more surviving individuals.

Allele frequencies at all but one locus were not different from those expected under HW equilibrium ($P = 0.25$–0.77), but at locus *Hg8.9*, allele frequencies deviated significantly from equilibrium values ($\chi^2 = 10.47$, df = 4, $P = 0.03$). The distribution of alleles at this locus was also unusual, with the smallest 2 alleles common in both groups (>20% frequency), and many larger sized alleles found in single or a few individuals (see Supplementary Material). We conducted all subsequent analyses both with and without data from this locus. Of 66 pairwise comparisons, following Bonferroni correction for multiple tests, no pair of loci showed significant linkage disequilibrium in the total sample.

Heterozygosity at these 12 markers was high in most individuals and did not differ between the 2 groups (identical mean multilocus $h = 0.83$ in both survivors and nonsurvivors). The locus that was not in HW equilibrium (*Hg8.9*) was least heterozygous (0.71), whereas heterozygosity estimates for the other loci ranged from 0.74 for *Hg3.7* and *Hg6.3* to 1.0 for the most variable locus *Hl15*. Omitting locus *Hg8.9* reduced the variability in heterozygosity estimates for both survivors and nonsurvivors, but the pattern was unchanged (data not shown).

Eight of the 12 loci had higher d^2 estimates (substantially higher for 5 loci) in the surviving individuals, whereas only 1 (*Hg8.9*, not in HW equilibrium) showed the reverse trend (Figure 1A). Mean d^2 values ranged from 2.2 to 37.1 overall, with a mean value of 16.14 in the nonsurvivors and 19.02 in the survivors. This difference was not significant ($t = -1.22$, df = 61, 1-tailed $P = 0.11$), but the nonsurvivors had mean d^2 values clustered at the low end of the range, whereas the highest values were only seen in survivors (Figure 1B). Omitting locus *Hg8.9* made this trend stronger (data not shown), and the difference approached statistical significance ($t = -1.59$, df = 60, 1-tailed $P = 0.06$).

When mean d^2 values were standardized (dividing by the maximum value observed at each locus to reduce the influence of highly polymorphic loci such as *Hl15*; Hedrick et al. 2001), the 12 markers provided a more uniform signal, but the pattern of values for survivors and nonsurvivors was unchanged (Figure 2A). Combining these standardized values over all 12 loci showed a nearly statistically significant difference between nonsurvivors (mean = 0.143) and survivors (mean = 0.169; $t = -1.44$, df = 61, 1-tailed $P = 0.08$; Figure 2B). When locus *Hg8.9* was omitted, the difference reached significance (nonsurvivors: mean = 0.142; survivors: mean = 0.176; $t = -1.77$, df = 59, 1-tailed $P = 0.04$; data not shown).

IR values ranged from −0.19 to 0.33, with no significant difference between survivors (mean = 0.018) and nonsurvivors (mean = 0.005; $t = -0.41$, df = 62, 1-tailed $P = 0.34$), although the trend was in the opposite direction to that expected (survivors were apparently derived from more related parents than nonsurvivors). Omitting locus *Hg8.9* made this unexpected trend even stronger (mean for nonsurvivors: −0.009; survivors: 0.014).

Discussion

All the individuals in this study were stranded animals in poor health, outside their normal range. Some recovered and were released, whereas others did not survive. Although some of the deaths may appear to be accidental mortalities (and therefore not associated with fitness), nearly all harp seals found in Long Island waters during this time were juveniles in very poor condition. The ingestion of sand and rocks on local beaches appears to be a result of the species' habit of ingesting snow, their normal haul-out substrate in the Arctic. Because most of the nonsurvivors (including the possible boat-strike victim) could potentially have recovered (either before or after rescue), we consider the comparison of this group with the survivor group to be an instructive one.

Acevedo-Whitehouse et al. (2003) evaluated microsatellite diversity in stranded California sea lions (*Zalophus californianus*) and found that sick animals had higher estimated parental relatedness (IR values) compared with control

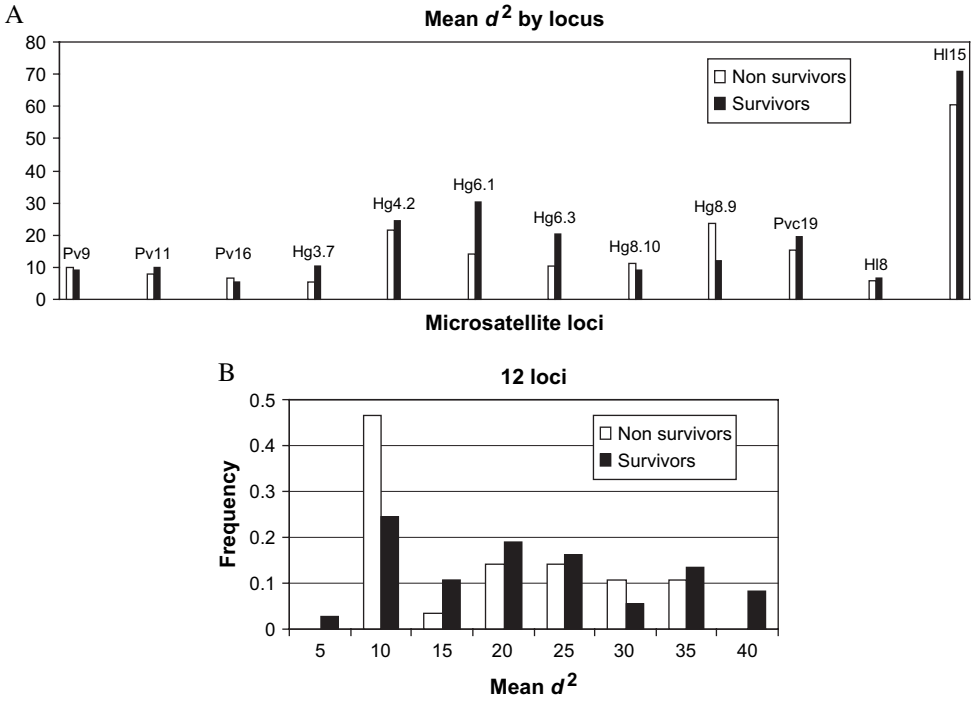

Figure 1. Mean d^2 values in surviving and nonsurviving stranded harp seal juveniles (**A**) at each individual locus and (**B**) frequency histogram of mean values over all 12 loci.

animals affected by trauma. Similarly, a study of gray seal (*H. grypus*) pups demonstrated significantly higher microsatellite diversity in healthy versus dead individuals, and those dying from trauma and malnutrition had the lowest IR values among the dead pups (Bean et al. 2004). Our sample of harp seals had no such healthy control individuals, and the timing of rescue was likely a very important determinant of rehabilitation success, potentially masking any existing genetic

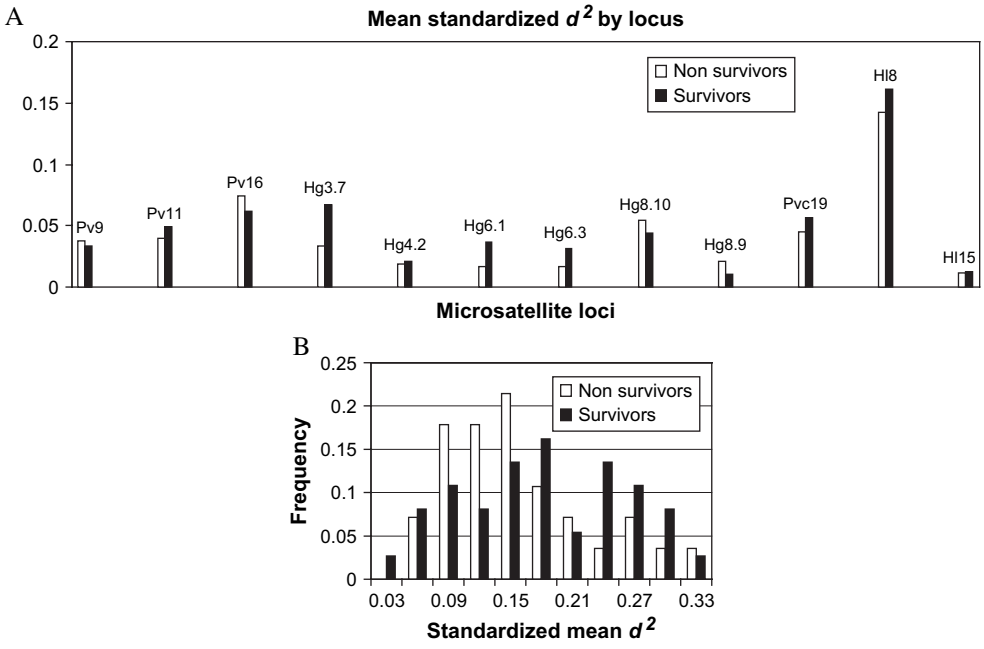

Figure 2. Standardized mean d^2 values (uncorrected value divided by maximum value observed at each locus) in surviving and nonsurviving stranded harp seal juveniles (**A**) at each individual locus and (**B**) frequency histogram of mean values over all 12 loci.

predisposition for survival. Therefore, the finding that surviving individuals tended to have higher mean d^2 values than nonsurvivors (Figures 1 and 2) indicates that this may be a very sensitive measure of fitness under some conditions.

These studies of other pinnipeds utilized some of the same microsatellite markers we employed, but genetic variation was higher in our harp seal study. For example, Bean et al. (2004) used 9 markers in more than 1000 gray seal samples and found only 6–11 alleles per locus, with heterozygosities ranging from 0.56 to 0.84. In our study, at least 9 alleles per locus were detected in a much smaller number of individuals, and heterozygosities ranged from 0.71 to 1.00. The same locus (*Hg8.9*) that was least heterozygous in our sample of 65 harp seals was the most variable marker in the gray seal study (Bean et al. 2004). Similarly, in the Coltman et al. (1998) study of 258 harbor seal pups, only 5 alleles were detected at locus *Pv11* (vs. 11 in our study) and only 2 alleles at locus *Pvc19* (vs. 14 in our study).

The highly variable nature of these microsatellite markers in harp seals may explain why our estimates of IR did not show the expected pattern of lower values (indicative of more outbred individuals) for the surviving seals. Because our data set included so few homozygotes, standardized mean d^2 estimates appeared to provide a more sensitive measure of microsatellite diversity in this case. In our study, 8 of 12 microsatellite loci showed the expected pattern of higher mean d^2 values in the survivors compared with the nonsurvivors (Figures 1A and 2A). The only locus which showed a substantial difference in the opposite direction (*Hg8.9*) was also the only locus that clearly did not conform to HW equilibrium allele frequencies.

Our data show that microsatellite mean d^2 estimates may provide an effective index of fitness in some species with large population size and high genetic variation. Under these circumstances, standardized heterozygosity and IR measures may be less informative, as the data set contains very few homozygotes. Although all the juvenile harp seals sampled for this study were in poor condition on initial stranding, those that recovered had slightly higher mean d^2 values than those that died, but they did not have lower IR values. Future research should include mapping these microsatellite loci on harp seal chromosomes to evaluate linkage patterns and an examination of variation at fitness-related loci such as the major histocompatibility complex (MHC). Increased variation at MHC loci has been associated with increased disease resistance and improved juvenile survival in other species (e.g., sheep *Ovis aries*; Paterson et al. 1998) and might explain the ability of some of these harp seals to recover from illness better than others.

Supplementary Material

Genotypes at 9–12 microsatellite loci for 28 nonsurviving and 37 surviving stranded harp seal juveniles and mean d^2 and IR values provided for each, as well as the cause and timing of stranding/death or stranding/release, if known, are available as supplementary material at http://www.jhered.oxfordjournals.org/.

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