Estimation of effective population size (N_e) in an island population of feral horses (*Equus caballus* L.)

By

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ABSTRACT

A concern of conservation biology is the viability of small, isolated populations because these are expected to lose genetic diversity at a greater rate than large populations due to genetic drift. Factors such as non-random mating systems and population fluctuations are known to reduce genetic diversity in natural populations. The Sable Island Horse (*Equus caballus* L.) population exhibits these characteristics; as such it is an interesting population in which to explore change in genetic diversity over time. Effective population size (N_e) is the size of a Wright-Fisher ideal population exhibiting the same level of genetic drift as the study population. Comparison of N_e with census population size can be informative about the rate at which genetic diversity is changing in the study population, and about what factors are likely influencing patterns of genetic diversity. This study aimed to use the Sable Island Horse population as a case study of how traits known to reduce genetic diversity affect estimates of N_e. Heterozygosity (±SE) of the population based on samples collected in 1987-88 was 0.560 (±0.020). By 2011 heterozygosity had declined to 0.536 (±0.018). The maximum range of N_e estimates was 20 to 244 individuals with 2011 estimates averaging 60.9 individuals. This is well below the census size of 150 to 400, which is consistent with expectations given the size, lack of migration, non-random mating system, and population fluctuations of the horse population. It seems that the population has not yet reached mutation-drift equilibrium. Areas for future investigation include developing a pedigree of the horses and deeper investigation of the mating system, particularly in regards to mate choice and relatedness in harem bands.

Date: April 17, 2013

INTRODUCTION

A concern of conservation biology is the viability of small, isolated populations (Palstra & Ruzzante 2008). Genetic diversity is what enables a population to adapt to changing environmental conditions by providing the raw material on which natural selection can act (Frankham *et al.* 2004; Hedrick 2011). As such, populations with greater genetic diversity are better able to adapt to adverse conditions and/or take advantage of new opportunities in the environment. This evolutionary potential can help ensure the long term viability of a population (Anthony & Blumstein 2000).

Small populations are expected to lose genetic diversity at a greater rate than large populations due to genetic drift (Hedrick 2011). Genetic drift is the chance change in allele frequencies due to the stochastic nature of gametic sampling, resulting in the random loss or fixation of alleles (Frankham *et al.* 2004). The magnitude of genetic drift is increased by isolation, a non-random mating, and population fluctuations (Anthony & Blumstein 2000; Hedrick 2011).

A population that is isolated undergoes no gene flow with other populations. Because no new alleles are introduced by immigration, the only source of new genetic variation is mutation (Frankham *et al.* 2004). When the force of genetic drift is stronger than the rate of mutation, genetic diversity will decline as alleles will drift towards fixation or loss at a greater rate than the generation of new alleles by mutation (Hedrick 2011).

Non-random mating can have an impact on the rate of decline of genetic diversity in two ways; through skewed mating systems and inbreeding. In a population with a skewed mating system, such as polygyny, one sex may have a high variance in reproductive success (Stiver *et al*. 2008). A proportion of the members of this sex may have little or no opportunity to pass their genes to the next generation, resulting in a loss of genetic diversity in a population over time. In this way, a skewed mating system reduces the effective gene pool of a population, and subsequently increases the rate of genetic drift above what would be expected if all reproductively available individuals reproduced (Stiver *et al*. 2008; Storz 1999).

Inbreeding occurs when mating individuals are more closely related to each other than is expected by chance (Szulkin *et al.* 2010). Inbreeding does not result in a change in allele frequencies but rather a change in the organization of alleles in a genotype; the proportion of individuals homozygous by descent in population increases (Keller & Waller 2002). One measure of genetic diversity is the degree of heterozygosity of a population (Hedrick 2011); therefore inbreeding directly contributes to the loss of genetic diversity by reducing the heterozygosity of a population. Inbreeding may have fitness effects on the population as homozygous individuals often have lower rates of survival and reproduction than heterozygous individuals, a phenomenon termed 'heterozygote advantage' (Keller & Waller 2002). This can result in inbreeding depression, the reduced reproductive fitness of inbred individuals relative to outbred individuals (Keller & Waller 2002; Szulkin *et al.* 2009).

Population fluctuations, or bottlenecks, occur when a population undergoes a severe reduction in numbers, followed by population expansion (Hedrick 2011). Individuals from the pre-bottleneck population that do not survive to reproduce do not make a genetic contribution to subsequent generations. Only the genes of the survivors, which may represent only a fraction of the genetic diversity of the pre-bottleneck population, are passed on. One type of bottleneck is the founder effect (Hedrick 2011). This occurs when a small number of individuals found a new population. Only the alleles present in the founders may be passed on to their descendants. As such, bottlenecks, whether due to periodic reduction and expansions or due to the founder effect, result in a reduction in genetic diversity in a population (Wright 1969; Vucetich *et al.* 1997).

The Sable Island Horses

Plante *et al.* (2007) characterized the genetic diversity of the Sable Island horse population in comparison with 20 other horse breeds. They found that this population had lower genetic diversity than these breeds as well as other populations of feral horses (Plante *et al.* 2007). Furthermore, it was found that the Sable Island horses have

significantly diverged from ancestral breeds. Loss of the Sable Island population would result in a greater decrease in diversity at the breed level than the loss of any other horse population included in the study (Plante *et al.* 2007). As such, they concluded that the Sable Island horses are a unique breed and may be an important genetic (Plante *et al.* 2007).

The Sable Island horses exhibit many of the characteristics that are expected to contribute to genetic drift and erode genetic diversity in a population over time. The population originated with a small number of individuals who were placed on the island in the mid-1700s (Lucas *et al.* 1991), creating a founder effect. Since then the population has undergone a number of expansions and contractions, fluctuating between approximately 150 and 400 individuals (Lucas *et al.* 1991). This has the effect of creating serial bottlenecks, from which only a reduced proportion of individuals remain to pass genetic material to the next generation. With each contraction in population size genetic diversity is expected to be reduced (Vucetich *et al.* 1997).

Sable Island is a crescent sand bar located approximately 160km from the nearest landfall (Parks Canada 2012); therefore, without interference, the population is completely isolated from gene flow. While new breeding horses were introduced to the island throughout the 1800s and early 1900s the population has been unmanaged and legally protected from interference since 1960 (Lucas *et al.* 1991; Lucas *et al.* 2009; Plante *et al.* 2007). As no new individuals are introduced to the population immigration is not a source of new alleles.

The horses have a polygynous mating system which is a non-random mating system (Lucas *et al.* 1991). One male, the band stallion, may have a harem of one or several mares (Lucas *et al.* 2009). Males who do not have a harem form bachelor bands (Lucas *et al.* 1991; Lucas *et al.* 2009). Some of these males may never have the opportunity to breed. As such, the males may pass their genetic material to subsequent generations unequally and the potential genetic contribution of many males may be lost.

Another aspect of non-random mating among the horses is inbreeding. A study by Lucas *et al.* (2009) examined population substructuring and levels of inbreeding along the length of the island. They found genetic evidence of inbreeding, though levels of inbreeding vary, forming a gradient along the length of the island (Lucas *et al.* 2009).

Effective Population Size (Ne)

It can be difficult to detect the effects of each of these factors independently in natural populations, to determine exactly which evolutionary forces may be at work in a population, or to determine the relative contribution of each of these factors to genetic decline in a population (Palstra & Ruzzante 2008). The effective population size (N_e) is a theoretical metric that has been developed to capture the impacts of all of these forces on patterns of genetic variation over time (Leberg 2005). Effective population size is the size of an ideal Wright-Fisher population that would exhibit the same amount of drift as the population under study (Wright 1969). An ideal population is one in which the sex ratios are equal, mating is random, generations are discrete, and selection, gene flow, and mutation are negligible (Wright 1969). There are very few, if any, natural populations that conform to these assumptions (Leberg 2005). However, identifying how census data compare to these theoretical expectations provides information on how, and to what degree, the biology of the population (e.g. mating system, selection, and other factors mentioned above) is influencing genetic diversity (Palstra & Ruzante 2008). Thus, estimating N_e is a useful means to assess the impacts of complex, and difficult to study, factors into one metric that can be estimated somewhat easily (Palstra & Ruzzante 2008). Moreover, the size of the N_e directly determines how quickly genetic diversity will be lost from a population (Leberg 2005). Therefore, once an estimate of N_e is available, it is possible to make inference on the potential rate of this genetic loss and therefore identify if, and to what degree, conservation efforts are needed to mitigate this loss (Anthony & Blumstein 2000).

There are a number of methods used to estimate N_e and each is more or less robust to violations of and deviations from the Wright-Fisher ideal (Leberg 2005; Wang 2005). Earlier estimators of N_e are temporal estimators (Beebee 2009). That is, they require sampling from more than one time period spaced one or more generations apart and use the degree of genetic diversity lost during that time to obtain and estimate of N_e (Leberg 2005). However, it may not be feasible to obtain samples from natural populations with the requisite temporal spacing, particularly in species with a long generation time (Beebee 2009). As such, a number of methods have been developed that require data from only a single generation to estimate N_e. These methods examine particular parameters or characteristics of a population in a single generation and estimate the population size in an ideal population that would exhibit these parameters (Tallmon *et al*. 2008; Wang 2009; Waples & Do 2008).

The Wright-Fisher model predicts the change in heterozygosity in a finite, ideal population due to genetic drift and increasing common ancestry (Wright 1969). The Wright-Fisher model shows that heterozygosity declines at a rate of $1/2N_e$ per generation (Wright 1969). As N_e increases, $1/2N_e$ becomes very small, therefore $1-1/2N_e$ approaches 1 and loss of heterozygosity due to genetic drift declines. Inversely, as N_e becomes smaller, loss of heterozygosity due to genetic drift accelerates. Thus, assessing changes in heterozygosity over time provides one approach to estimating N_e (Wright 1969)

Another method that uses temporal data examines the standardized variance in change in allele frequencies over time to estimate N_e (Palstra & Ruzzante 2008). Similar to heterozygosity, allele frequencies in an ideal population change at a predicted rate, and therefore the amount of observed change over time can be used to estimate N_e (Kimura & Ohta 1969). Again, random changes in allele frequencies are expected to be greater in smaller populations than in larger populations as variance in allele frequencies is inversely related to population size (Palstra & Ruzzante 2008).

A method has been developed to use sibship assignment to estimate N_e from a sample drawn from a single generation (Wang 2009). It estimates N_e as a function of the

frequencies of full sib, half sib and non-sib dyads in a sample. The smaller the N_e the more likely it is two individuals drawn at random from a discrete generation will be full or half sibs (Wang 2009). As N_e increases in size, the probability that two individuals drawn at random share one or both parents declines. This method assumes discrete generations; that is, that all individuals in the sample are from the same generation and that the sample does not include individuals from the parental generation. If this assumption is violated, parent-offspring relationships may be incorrectly inferred to be full-sibling relationships (Wang 2009).

Approximate Bayesian computation may be used to estimate N_e from a single sample (Tallmon *et al.* 2008). This method generates a large number of simulated populations, with a range of N_e values, and compares selected summary statistics of the simulated populations to the same statistics in the study population. In this way a wide range of potential values of N_e can be narrowed down to a smaller probable range of N_e. Summary statistics may be selected based on parameters that are known to vary with or influence N_e (Tallmon *et al.* 2008). Knowledge of the summary statistics used in a particular computation may be informative about the influences on a particular population's N_e.

Linkage disequilibrium is the non-random association of alleles at two loci in gametes within a population (Lewontin & Kojima 1960). It can result from physical linkage, where alleles at multiple loci are inherited together because they are close together on a chromosome (and thus inherited as a single unit); or if selection favours certain combinations of alleles, so that specific allelic combinations are found together more often than expected if the alleles were not associated with one another (Slatkin 2008). The factors that reduce N_e, (including genetic drift, inbreeding, and bottlenecks) can result in multiple loci occurring together more often than expected, and thus the appearance of linkage disequilibrium (Hedrick 2011). As such, methods that examine linkage disequilibrium in a population have been used in the estimation of N_e (Waples 2006; Waples & Do 2010) Linkage disequilibrium varies inversely with N_e. Therefore,

as N_e declines, linkage disequilibrium increases and as N_e increases, linkage disequilibrium declines (Hedrick 2011).

Microsatellite Analysis of Populations

Microsatellites are stretches of DNA with a repeating sequence, such as a di-, tri-, or tetranucleotide sequence, repeated sequentially in tandem (Ellegren 2004). Microsatellites are not subject to natural selection as they reside in the non-coding regions of the genome (Ellegren 2004). Microsatellites mutate at a rate much higher than base pair substitutions, insertions, or deletions (Levinson & Gutman 1987). This is due to error-prone nature of the replication of repeating sequences; in a process known as slipped-strand mispairing, loops of the template or replicate strand of the DNA may form resulting in a decrease or increase in the number of repeats in the replicate microsatellite (Levinson & Gutman 1987). When this mutation occurs it most often results in a change of a single repeat unit, but may occasionally result in a change of more than one repeat unit (Ellegren 2004). Their high mutation rate also means that microsatellite loci are highly polymorphic (Ellegren 2004). Due to their neutral and rapidly mutating nature, microsatellites are ideal for observing genetic change and trends in populations over relatively short periods of time such as generations (Hedrick 2011).

Study Goal and Objectives

As a small, isolated, and genetically differentiated population, the Sable Island Horse population may be vulnerable to environmental changes due to loss of genetic diversity. As low genetic diversity leaves a population less able to adapt to environmental changes, this potential decline may have implications for the long-term viability of the population. However, it is not currently known at what rate genetic diversity is changing. Estimates of N_e can provide an indication of the rate of decline of genetic diversity. As such,

knowledge of N_e of the Sable Island horse population can provide an indication of the viability of the population now and into the future.

This goal of this study was to use the Sable Island Horse population as a case study of how traits known to lower genetic diversity, such as small population size, isolation, non-random mating, and inbreeding affect estimates of N_e in a wild population. This study had three objectives: to characterize genetic diversity, as measured by heterozygosity, of the population; to estimate the effective population size (N_e) of the population; and, to use estimates of effective population size to infer the rate of loss of heterozygosity in the population.

MATERIALS AND METHODS

Sampling

As part of long-term surveys of the Sable Island Horse population tissues samples have been collected from deceased horses on the island since 1987. Skin and muscle samples from each carcass were stored in salt-saturated 20% DMSO (dimethyl sulfoxide) solution (Seutin *et al.* 1991). To understand the change in heterozygosity over time, samples from all 55 horses that died in 1987-1988 (the earliest years for which samples are available) and all 77 horsed that died in 2011 have been selected for analysis, for a total of 132 samples. Two years were chosen for the "starting time" to result in a sample size of at least 50 individuals and a sample size similar to that for 2011. At an average generation interval of 4 years (Plante *et al.* 2007), this 24 year separation between sampling periods separates these samples by six generations.

DNA Extraction and Quantification

Forty milligrams ± 5mg of tissue were isolated for extraction. Where available, skin tissue was used but for some samples only muscle was available. Skin was minced with a scalpel then ground (by mortar and pestle chilled with liquid nitrogen) in 100µL of lysis buffer (10mM Tris (pH 8.0), 10mM EDTA (ethylenediaminetetraacetic acid, pH 8.0), 2%

SDS (sodium dodecyl sulfate), 0.1M NaCl, 40mM DTT (dithiothreitol), then added to 300µL lysis buffer for a total of 400µL of lysis buffer. Muscle tissue was minced with a scalpel and placed directly into 400µL of lysis buffer. An equivalent weight of beef tissue in 400µL of lysis buffer was used as a positive control and 400µL of lysis buffer alone was used as a negative control for all extraction procedures. All samples were incubated for one week at room temperature, upended daily to mix the solution. After one week incubation 0.5U of Proteinase K per milligram of tissue (33.3µL of 600U/mL proteinase K) was added to the samples. Samples were incubated overnight at room temperature after which time an additional 33.3µL of proteinase K was added to each sample. Samples were then incubated in a 65^{o}C waterbath for one hour then left to cool slowly to room temperature in a tray of water from the 65^{o}C waterbath for one hour. A final 33.3µL of proteinase K was added to the samples, which were then incubated at room temperature overnight.

An organic phenol:chloroform extraction was used to separate the DNA from the lysed cell materials (Sambrook & Russell 2001). An equal volume (400µL) of phenol:chloroform was added to each sample. Samples were upended for five minutes then centrifuged at 12,000xg for 2 minutes. The top aqueous layer was pipetted into new tubes and the bottom organic layer discarded. This process was repeated twice, once using phenol:chloroform, a second time using choloroform.

Ethanol precipitation was used to concentrate the recovered DNA. Briefly, ammonium acetate was added to the samples to a concentration of 2M. Two times equal volume (800µL) of ice cold 95% ethanol was added to each sample. Tubes were flicked vigorously by hand to precipitate the DNA. Samples were then stored at -20^{o}C overnight.

The samples were centrifuged at 12,000xg for 10 minutes and the supernatant decanted. One hundred µL of 70% ethanol was added to each sample followed by centrifugation at 12,000xg for 10 minutes. Ethanol supernatant was decanted from the samples and excess ethanol removed with a Kimwipe. Samples were left to air dry 15 minutes. One hundred

fifty µL of $TE_{0.1}$ (20mM Tris-HCL, 0.1mM EDTA (ethylenediaminetetraacetic acid), pH 8.0) was added to each sample to suspend the pellet of DNA.

Samples were quantified by spectrophotometry using a NanoDrop 2000. Calf thymus standards of known concentration (1ng/ µL, 5ng/ µL, 10ng/ µL, and 50ng/ µL) were quantified along with the horse samples to ensure that the equipment was returning accurate readings. To double-check concentrations, and to assess the degree of DNA degradation, 20ng of DNA for each sample were electrophoresed through 2% agarose gels and visualized with SYBR Green stain. To determine the actual quantity of DNA in the gel for each sample the intensity of the UV illuminated bands of the extracted DNA, positive control, and negative control were compared with a low mass ladder that contains bands of 1, 2, 4, 8, 12, and 20ng of DNA respectively. The ratio of actual to expected (20ng) quantity of DNA in the gel for each sample was used to determine the functional concentration of DNA in the extracted samples. The quality of the DNA was assessed by eye by examining the degree of fragmentation of the electrophoresed DNA. High quality DNA produces a clear, tight band. Fragmented DNA appears as a smear along the length of the gel.

Microsatellite Genotyping

All samples were amplified at 17 microsatellite loci (VHL20, HTG4, AHT4, HMS7, HTG6, AHT5, HMS6, ASB23, ASB2, HTG10, HTG7, HMS3, HMS2, ASB17, LEX3, HMS1, CA425) using the Life Technologies StockMarks® Equine Genotyping Kit (Life Technologies, Dimsoski 2003). An 11µL reaction volume was used as follows: 1.61µL StockMarks PCR Buffer, 2.57µL dNTP mix, 0.32µL AmpliTaq Gold® DNA Polymerase, 2.57µL Amplification primer mix, 1.93µL reaction water, 2µL of 0.5 ng/µL DNA. Two µL of a 1:80 dilution of the control DNA provided in the kit was used as a positive control, 2µL TE0.1 was used in the negative control. The reaction underwent the following thermal cycling in an Applied Biosystems Veriti® 96-well thermal cycler: 10 minutes at 95^{o}C; 30 cycles of 30 seconds at 95^{o}C, 30 seconds at 60^{o}C, 60 seconds at

72°C; 60 minutes at 72°C; held upon completion at 4°C. Once the PCR was complete the amplified samples were stored at -20°C.

Amplified samples were prepared for size-separation by adding 2µL of PCR product to 10µL of Formamide and 0.25µL GeneScan™ – 600 LIZ® Size Standard (Applied Biosystems). Prepared samples were size-separated and visualized on an Applied Biosystems 3500xl Genetic Analyzer. The microsatellite alleles were scored with GeneMarker® (SoftGenetics) software. Raw data files were downloaded from the Genetic Analyzer and uploaded to GeneMarker. The size standard for all samples was visually inspected to ensure that it was consistent and correct within and across each sample. A panel was created for the equine loci. For each locus amplified by the StockMarks kit a series of electropherograms of the sample data was inspected to determine appropriate allele bins. This was done by examining the peak patterns and selecting allele bins at two base pair intervals (as all loci amplified by the StockMarks kit have dinucleotide repeats).

The GeneMarker software made allele calls based on the panel that was developed. All allele calls made by the software were verified and adjusted where necessary by eye. For instance, Taq polymerase may sometimes add an extra adenine to the end of an amplified segment (adenylation) creating a 'plus-A' peak. This may not happen evenly or consistently within a locus. As such, for some samples the 'allele' peak may be highest while in other samples the 'plus-A' peak may be highest. The software selects the highest peak as the allele. For instance, when the allele bins for a particular locus are selected to choose the 'allele' peak, GeneMarker may call the 'plus-A' peak as the allele if it has a stronger signal, and therefore indicate that the peak is 'off-ladder'. The software may occasionally make other incorrect calls, such as a homozygote incorrectly called as a heterozygote, a heterozygote incorrectly called as a homozygote, or three peaks called at a locus. Such artefacts must be manually adjusted.

Once all allele calls were made, verified, and adjusted where necessary, a report was exported from GeneMarker. This report took the form of a table listing each sample and

associated allele calls at each locus. Alleles were reported as allele size in nucleotides. This report formed the basis for all further analyses.

Calculation of Heterozygosity

A test of deviation from Hardy-Weinberg equilibrium was performed at each locus using the CERVUS 3.0 software (Marshall *et al.* 2008; Kalinowski *et al.* 2007). The overall observed heterozygosity for each time period was estimated by averaging the observed heterozygosity for each individual. A Wilcoxon Rank Sum test was performed in R (R Core Team 2013) to test for difference in heterozygosity between the two time periods.

Estimation of Effective Population Size

Two temporal estimations and three point estimations of effective population size (N_e) were performed. The temporal estimations were the Wright-Fisher model and the Moments-Based Temporal estimator developed by Waples (2009) as implemented in NeEstimator (Ovenden *et al.* 2007). Data from 1987-88 and 2011 were used to estimate a single N_e for each of these models. The point estimates were sibship assignment as implemented in COLONY Version 2.0 (Wang 2009); approximate Bayesian computation as implemented in ONeSAMP (Tallmon *et al.* 2008); and a linkage disequilibrium method as implemented in LDNe (Waples & Do 2008). For each of these models data from both 1987-88 and 2011 were used to generate two estimates of N_e for each model. As such, it was possible to compare current and historic estimates of N_e for the Sable Island Horse population.

The Wright-Fisher model is based on the equation: $H_t = (1 - {}^{1}/_{2Ne})^{t} H_o$; where *Ht* is heterozygosity after *t* generations, H_o is the heterozygosity at the initial time period, and N_e is the effective population size (Hedrick 2011; Wright 1969). The value for H_o was the estimated heterozygosity in 1987-88, H_t was the estimated heterozygosity in 2011, and *t*

was 6 generations. A time period of six generations was selected as the horses have an average generation time of 4 years and 24 years passed between sampling periods.

The Moments-Based Temporal estimate was calculated using the program NeEstimator (Ovenden *et al.* 2007) based on the work of Waples (1989) which in turn was developed from earlier temporal methods of calculating N_e such as that of Nei & Tajima (1981). The size of the population varies inversely with changes in allele frequency; as N_e becomes smaller, greater changes in allele frequencies are expected to be observed over time. This method estimates N_e by measuring changes in allele frequencies between samples taken one or more generations apart (Nei & Tajima 1981; Waples 1989). Genotype data from 1987-88 and 2011 were input for use in this program. NeEstimator reports the calculated N_e along with a 95% confidence interval.

The Sibship Assignment estimate was calculated in the program COLONY Version 2.0 (Wang 2009). Population size is expected to vary inversely with relatedness. A smaller population will have a higher proportion of full and half sibs; a larger population will have a smaller proportion of sibs. COLONY employs multilocus genotypes of a single cohort of a population to assign sibship to pairs of individuals. This is done by implementing full-pedigree likelihood methods (Jones & Wang 2010). Once sibship has been assigned, COLONY uses the frequency of full and half sibs to estimate N_e. COLONY reports the calculate N_e along with 95% confidence intervals (Jones & Wang 2010; Wang 2009). The genotype data for both 1987-88 and 2011 sample were input for use in this program.

The approximate Bayesian computation was performed by the program ONeSAMP (Tallmon *et al.* 2008). ONeSAMP employs eight summary statistics in the estimation of N_e (Tallmon *et al.* 2008): the number of alleles divided by allele length range (Garza & Williamson 2001), the difference of the natural logarithms or variance in allele length and heterozygosity (King *et al.* 2000); expected heterozygosity (Nei 1987); number of alleles per locus; Wright's F_{is} (Nei 1987), the mean and variance of multilocus homozygosity, and the square of the correlation of alleles at different loci (Hill 1981). These summary

statistics were selected as either population genetics theory or simulations conducted by the ONeSAMP developers have established a relationship between these statistics and N_e (Tallmon *et al.* 2008). ONeSAMP creates 50 000 iterations drawing a value for N_e from the prior (the range of possible N_e) provided by the user. Each simulation reproduces in accordance with the Wright-Fisher ideal for two to eight generations then summary statistics are calculated. These summary statistics are compared with the summary statistics computed from the study population data set. The prior is accepted and weighted or discarded according to the similarity or dissimilarity between these two sets of statistics. The final estimate of N_e is calculated from the weighted accepted values of N_e from the simulations. ONeSAMP reports the mean and median N_e along with a 95% confidence interval (Tallmon *et al.* 2008). The genotype data for the 1987-88 and 2011 samples were used as input for this program to generate an estimate of N_e for each time period. A prior of 2 to 800 was selected as 2 is the minimum value that the program accepts and 800 is twice the maximum census size of the population. This range should be sufficiently conservative as N_e is generally lower than population size (Tallmon *et al.* 2008).

The linkage disequilibrium based estimate was calculated by the program LDNe (Waples & Do 2008). They indicate that allele frequencies close to zero or one can affect estimates of N_e. As such, LDNe returns three estimates of harmonic mean sample size and N_e; one each that excludes all alleles below the critical values of 0.05, 0.02, and 0.01, with the option for the user to include up to three more critical values for a total potential of six critical values. They found that N_e estimates below the critical value of 0.05 bias the estimate of N_e upwards. As such, the estimate at the critical value of 0.05 was selected for analysis. LDNe provides 95% confidence intervals at the three critical values using both parametric and jackknife methods. The 95% confidence interval using the jackknife method was selected for analysis as Waples & Do (2008) indicate that the parametric method may provide confidence intervals that are too narrow. The program allows selection of one of two mating models: random mating or monogamy (Waples & Do 2008); random mating was selected.

Simulations

To determine whether the rate of change of heterozygosity in the Sable Island Horses was similar to that expected by classical population genetics models simulations were used. Simulations were conducted in EASYPOP (Balloux 2001). Three Wright-Fisher ideal populations were simulated for three different values of N_e. The N_e values chosen were the lowest 95% confidence limit generated among all five estimators, the highest 95% confidence limit generated among all five estimators, and the average of the 2011 and temporal estimates. Twenty iterations of each of these populations were run for 400 generations. The heterozygosity at each generation was averaged. This enabled characterization the expected change in heterozygosity across the range of estimates of N_e for the Sable Island Horse population as a function of time (in generations). Expected rate of change in heterozygosity may be estimated by examining the slope of the curve for a particular N_e at a given level of heterozygosity.

RESULTS

Amplification and Scoring of Microsatellite Loci

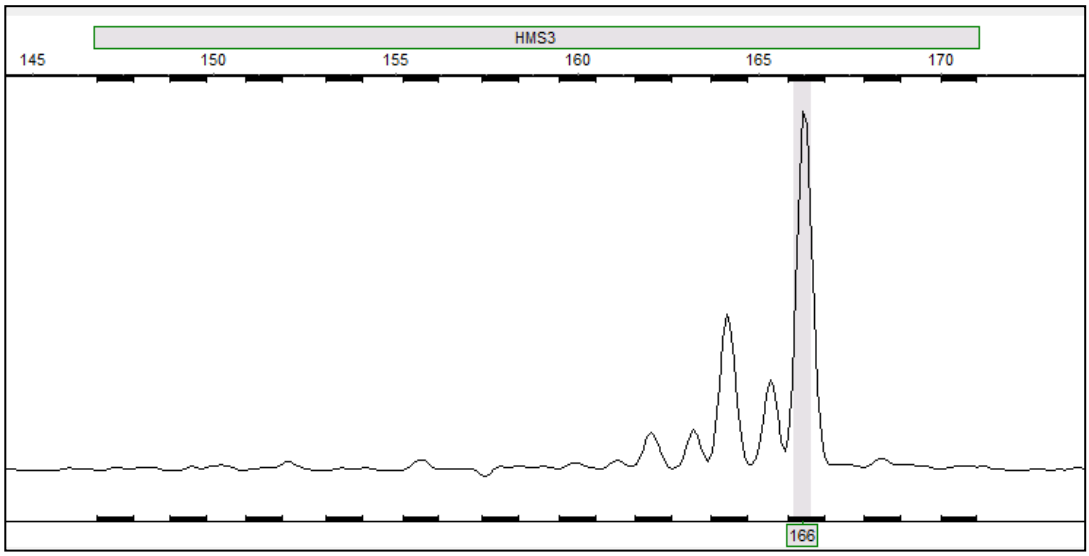
Of the 17 loci that were amplified with the StockMarks Equine Genotyping Kit five (HTG6, HMS6, HTG10, HMS3, HMS1) were discarded due to ambiguity in the interpretation of the electropherograms. It was deemed that these loci could not be reliably scored. Of the remaining 12 loci, LEX3 was removed from analysis as it was determined it did not satisfy the assumptions of Hardy-Weinberg equilibrium. Locus ASB23 was deemed by the ONeSAMP (Tallmon *et al.* 2008) to have too much missing data and was removed from all analyses. All results and subsequent analyses are therefore based on the remaining 10 loci (VHL20, HTG4, AHT4, HMS7, AHT5, ASB2, HTG7, HMS2, ASB17, CA425). Of the 55 samples from 1987-88 and the 77 samples from 2011, 51 and 75 samples respectively were successfully amplified (example in Figure 1) and were subsequently genotyped and used in analyses. The remaining samples

either failed to amplify or were deemed to have too much missing data when genotyped and were therefore excluded from analysis.

Estimation of Heterozygosity

Estimates of number of alleles, observed heterozygosity, and expected heterozygosity are summarized in Table 1. Estimates of heterozygosity for each of the two time periods were taken by averaging the heterozygosities of all individuals in the respective samples (Table 1). Heterozygosity (± se) in 1987-88 was estimated at 0.559 (0.020) while heterozygosity in 2011 was estimated at 0.536 (0.018). The p-value of the Wilcoxon Rank Sum test was 0.5433, indicating that the heterozygosity at the two sampling periods is not significantly different.

a)



b)

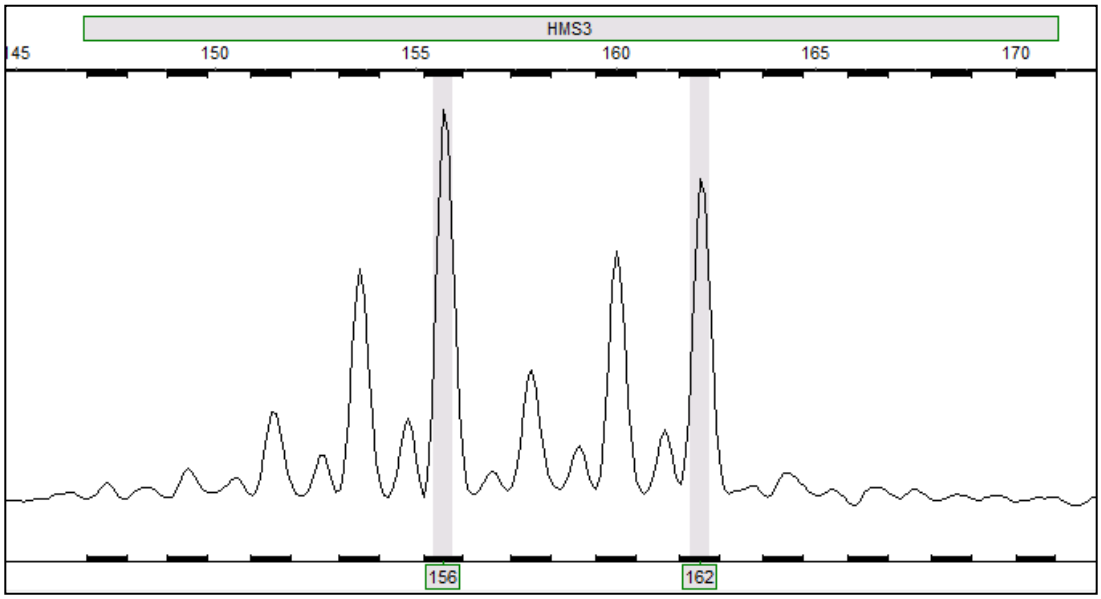


Figure 1

Sample electropherogram for a a) homozygote and b) heterozygote at locus HMS3. The vertical grey bars and the numbers below the electropherogram represent the 'allele calls' for the locus.

Table 1

Estimates of number of alleles (k), observed heterozygosity (Ho), and expected heterozygosity (He) by locus for Sable Island Horse Population in a) 1987-88 and b)2011.

a) 1987-88

Locus	k	N	Ho	He
HMS3	3	50	0.54	0.568
VHL20	5	51	0.608	0.674
HTG4	3	50	0.58	0.633
AHT4	5	51	0.765	0.691
HMS7	3	51	0.333	0.525
AHT5	3	48	0.563	0.621
ASB2	4	50	0.4	0.491
HMS2	2	47	0.128	0.121
ASB17	9	51	0.882	0.83
CA425	3	51	0.745	0.574

b) 2011

Locus	k	N	Ho	He
HMS3	3	67	0.493	0.59
VHL20	6	73	0.575	0.562
HTG4	3	74	0.703	0.666
AHT4	4	74	0.689	0.667
HMS7	3	75	0.507	0.585
AHT5	4	72	0.5	0.612
ASB2	4	75	0.373	0.354
HMS2	2	74	0.149	0.161
ASB17	9	75	0.76	0.833
CA425	3	75	0.587	0.517

Estimation of Effective Population Size

The maximum range of the estimates of Ne was from 20 (the lower 95% CI generated by sibship assignment) to 244 (the upper 95% CI generated by the Moments-based Temporal estimator) (Figure 2). The 2011 point estimates and the temporal estimates together averaged 60.9 individuals.

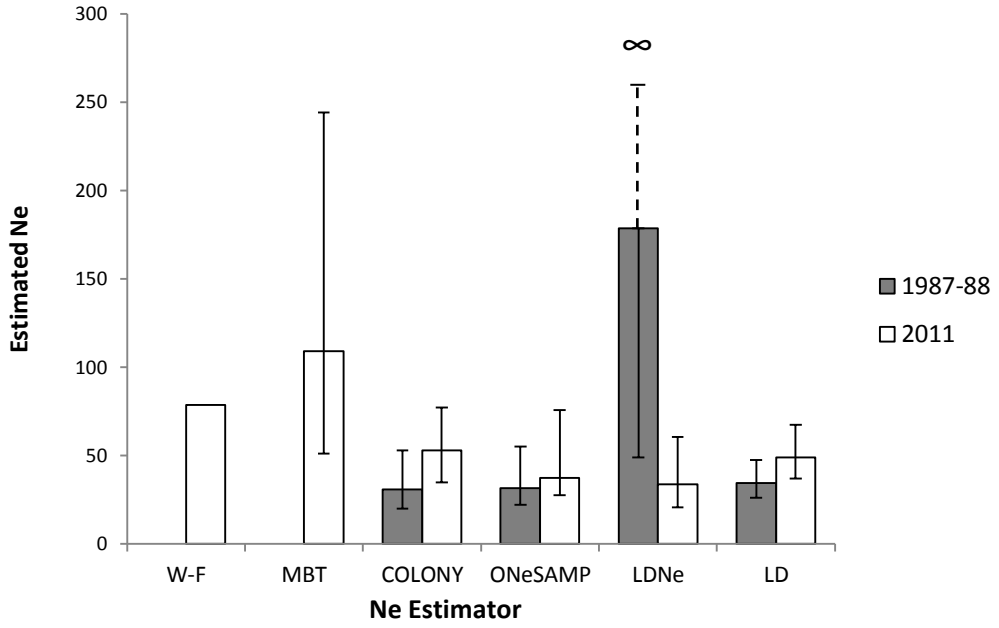


Figure 2

Effective population size of the Sable Island Horses (with 95% CI).
W-F = Wright-Fisher; MBT = Moments-based Temporal as calculated by NeEstimator; COLONY = Sibship Assignment as calculated by COLONY; ONeSAMP = Approximate Bayesian Computation as calculated by ONeSAMP; LDNe = Linkage Disequilibrium as computed by LDNe; LD = Linkage Disequilibrium as computed by NeEstimator. The Wright-Fisher and Moments-based Temporal models span 1987-88 to 2011. The COLONY, ONeSAMP, LDNe and LD models took estimates at both 1987-88 and 2011.

Simulations

The three values of N_e used for simulations of change in heterozygosity were 20, 244, and 60. The size of each population was held constant throughout the simulation. Heterozygosity in the population as of 2011 is indicated by the horizontal grey line (Figure 3). The rate of change of genetic diversity as of 2011 at each value of N_e may be approximated by the slope of the curve for each value of N_e at the intersection of this line.

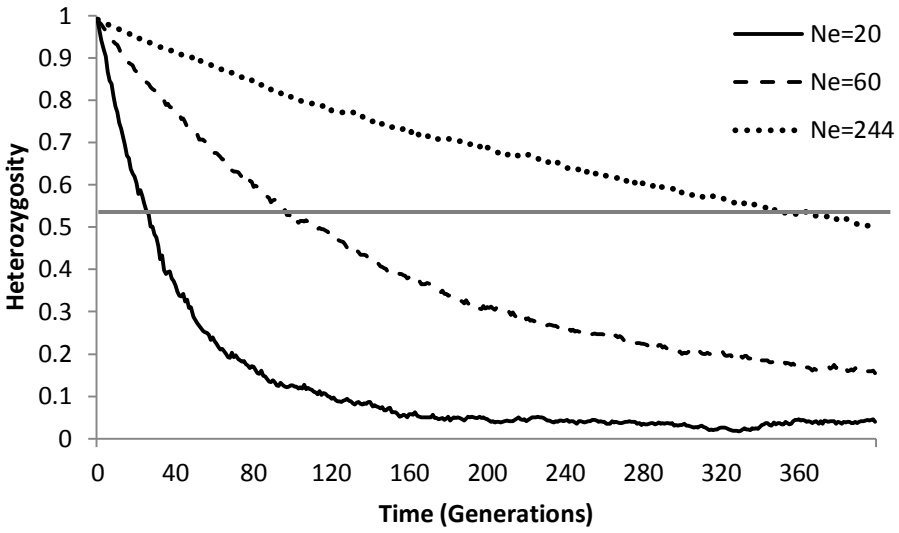


Figure 3

Expected decline in heterozygosity over time at effective population size of 20, 60, and 244. The horizontal grey line represents the heterozygosity of the Sable Island Horse population in 2011 (0.536). The slope of the curve for each N_e at the intersection of this line can be approximated to the current rate of change in genetic diversity for each N_e.

DISCUSSION

Of the ten estimates of N_e generated, eight estimates fall well below the census population size of 150-400. Low estimates of N_e relative to census size suggest that genetic drift is a strong force in this population (Leberg 2005). This is consistent with the traits in the population that are known to reduce genetic diversity and increase the magnitude of genetic drift over time.

The population is relatively small. As the population fluctuates between 150 and 400, and as the island has a finite amount of space and resources (Lucas *et al.* 2009), the upper size of this range may be the island's carrying capacity for the horse population (Plumb *et al.* 2009). As such the population may not be able to exceed this upper limit. Furthermore, the population is isolated. This means that declines in heterozygosity due to genetic drift cannot be counteracted by gene flow through contact with another

population (Frankham *et al.* 2004). This is reflected in the low estimates of N_e for the Sable Island Horse population.

Characteristics which may be more challenging to detect and interpret include population fluctuations and mating behaviour (Leberg 2005). The estimates of N_e, relative to actual N_e, may be downwardly biased due to fluctuations in the population. This is particularly true of the linkage disequilibrium method (Waples 2006). Immediately following a bottleneck the estimates of N_e may be downwardly biased for a few generations, even if the population is undergoing expansion (Waples 2006). As the Sable Island Horse population has undergone frequent and occasionally severe population fluctuations (Lucas *et al.* 1991; Zoe Lucas, personal communication), this bias may never be completely absent from estimations of N_e and actual N_e may be higher than estimates suggest. This may explain why the temporal estimation methods returned higher estimates of N_e than the point estimation methods; the point estimates may be more sensitive to recent fluctuations while the temporal methods may be more robust to these fluctuations (Waples & Yokota 2007). When using the Moments-based temporal model in population of changing size with overlapping generations, sampling biases are minimized when taking samples at least three to five generations apart (Waples & Yokota 2007). The present study took samples at six generations apart. As such, the estimates of N_e generated by the point estimates in the range of 50 to 60 individuals may underestimate actual N_e while the temporal estimates at approximately 80 to 110 may be closer to the actual N_e of the population.

The slight variation in the values of N_e between 1987-88 and 2011 may be due to the increase in sample size from 51 in 1987-88 to 75 in 2011 and may reflect a change in population size. However, there is considerable overlap in the confidence intervals of the estimates for both of the time periods; as such, this finding should be interpreted cautiously. Due to the violation of the assumptions of the models of Ne, confidence on estimates is low and a different sampling regimen would be required to test for statistical differences between the two sampling periods.

Randomly mating populations are expected to lose genetic diversity at a slower rate than polygynous populations (Storz 1999). Genetic studies of the mating behaviour of the horses may contribute to our understanding of the mating system of the population to N_e. With the advent and application of DNA profiling to studies of behaviour in natural populations, many assumptions about animal behaviour have been revisited (Reynolds 1996). For example, by examining the DNA of putative parents and offspring, species previously thought to have been polygynous have shown evidence of multiple-mating by females (Reynolds 1996). Studies of other feral horse populations, such as the Misaki feral horses, have found that approximately 15% of successful matings by females were with males other than the band stallion (Kaseda & Khalil 1996). The degree to which the Sable Island mares mate with males other than their band stallion is unknown. To determine the degree to which genetic behaviour reflects social behaviour further studies should be undertaken.

An increase in relatedness in a population can reduce N_e (Wang 2009). The low estimates of N_e relative to census population size, particularly as estimated by the sibship assignment method suggests that the population exhibits a certain level of inbreeding. This is consistent with the findings of Lucas *et al.* (2009) which detected local inbreeding in the Sable Island horse population.

The accuracy of some of the methods used to calculate N_e may be better with a more selective use of samples (Waples & Yokota 2007). This study used all samples collected from horses deceased in either 1987-88 or 2011. As such, each sample included individuals from all life stages and may therefore represent individuals from two or more generations. As one interest of this study was examining how the heterozygosity of the population has changed over time, this sampling scheme was used to achieve the maximum temporal separation of samples. However, the method of sibship assignment as implemented in COLONY assumes that individuals originate from a single generation (Wang 2009). Inclusion of individuals from the parental generation does not invalidate the estimate, but it does bias estimates of N_e downwards. This bias becomes more pronounced as a greater number of individuals from the parental generation are included

(Wang 2009). The assumption that a sample contains individuals from a single generation has been violated in this study, though the degree of this violation and the number of generations potentially included in the sample is unknown. For instance, from the 2011 sample, of the 77 individuals from which tissue was collected, 18 are described as young, juvenile, foal, born in 2010 or 2011, or half-born. At least three pairs of half-born foals and mothers are included in this sample, which clearly violates the assumption that a single generation has been sampled. Furthermore, as the average generation time is four years (Plante *et al.* 2007) and the horses often live to ten years of age (Zoe Lucas, personal communication), it is possible that up to three generations are represented in these samples. As such, the estimate of N_e generated by the sibship assignment method may be lower than actual N_e.

The upper confidence limit of infinity generated by LDNe for 1987-88 may result when the contribution of sampling error is greater than the signal of genetic drift in a genetic data set (Waples & Do 2010). This can occur when the N_e is very large or when limited data are available. This is interpreted by the LDNe software as $N_e = \infty$. In this case it may only be stated that there is no conclusive evidence that the population is not very large, however the lower bound of the confidence interval, in this case 49, may be a plausible lower limit of N_e (Waples & Do 2010). Again, a different sampling regimen may improve this estimate.

As can be seen from Figure 3, the heterozygosity of the Sable Island Horse population is higher than expected for even the highest N_e estimated in this study for a population at mutation-drift equilibrium. This might suggest that the heterozygosity of the population is still in decline and has not yet reached mutation-drift equilibrium. As the population was founded in the mid-1700s and the generation interval is approximately four years (Lucas *et al.* 1991; Plante *et al.* 2007), between 60 and 70 generations have elapsed in this population. It may be that not enough time has elapsed for the population to reach equilibrium.

Though heterozygosity and estimates of N_e may be low, the population is currently at the high end of its size range at close to 400 individuals (Zoe Lucas, personal communication). This suggests that the horses are currently exhibiting reproductive fitness and may be well adapted to the local environment despite low and declining genetic diversity. As such, the population does not seem to be currently in need of active management for conservation purposes (Frankham 2004). However, the population should be genetically monitored as the traits in the population that contribute to low N_e, such as its small size, isolation, and non-random mating system, are also the factors that put the population at risk of inbreeding depression and endangerment (Frankham 2004; Keller & Waller 2002).

There are a number of further studies that could be undertaken to better understand this population. For further investigations of N_e, studies that examine all individuals born in a particular year, rather than deceased in a particular year, may be undertaken. Such cohorts spaced one or more generation apart could be compared to determine how N_e is changing over time in this population. In conjunction with census population estimates from field notes, the N_e/N ratio and changes in this ratio over time may be investigated. This ratio may be useful in understanding various factors such as reproductive success, mating systems, and conservation and management programs on N_e (Hedrick 2011; Waples 2005). Furthermore, as the Sable Island Horse population is known to undergo sometimes severe fluctuations, this population may be informative about the relationship between N_e, change in N_e over time, and bottlenecks. Network analyses could be undertaken to examine the interactions of social structure and relatedness. For instance, it has been observed in other feral horse populations that both males and females disperse from the natal group (Khalil & Murakami 1999; Linklater & Cameron 2009). This is somewhat unusual, as among mammals generally one sex disperses while the other remains in the natal group (Gros *et al*. 2009). Understanding this behaviour in the Sable Island Horse population may advance our understanding of the behavioural strategies that may be working to affect genetic diversity in this population. Lastly, as tissue samples have been taken from every horse that has died on the island since 1987, it is possible to

build a pedigree for the horses spanning nearly three decades. This would be an invaluable resource for understanding behaviour and the influence of various evolutionary forces on small, isolated populations over a period of generations.

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