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Management and Conservation Article

Pedigrees and the Study of the Wild Horse Population of Assateague Island National Seashore

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ABSTRACT Recently, a number of papers have addressed the use of pedigrees in the study of wild populations, highlighting the value of pedigrees in conservation management. We used pedigrees to study the horses (*Equus caballus*) of Assateague Island National Seashore, Maryland, USA, one of a small number of free-ranging animal populations that have been the subject of long-term studies. This population grew from 28 in 1968 to 175 in 2001, causing negative impacts on the island ecosystem. To minimize these effects, an immunocontraception program was instituted, and horse numbers are slowly decreasing. However, there is concern that this program may negatively affect the genetic health of the herd. We found that although mitochondrial DNA diversity is low, nuclear diversity is comparable to that of established breeds. Using genetic data, we verified and amended maternal pedigrees that had been primarily based on behavioral data and inferred paternity using genetic data along with National Park Service records of the historic ranges of males. The resulting pedigrees enabled us to examine demography, founder contributions, rates of inbreeding and loss of diversity over recent generations, as well as the level of kinship among horses. We then evaluated the strategy of removing individuals (using nonlethal means) with the highest mean kinship values. Although the removal strategy increased the retained diversity of founders and decreased average kinship between individuals, it disproportionately impacted sizes of the youngest age classes. Our results suggest that a combined strategy of controlled breeding and immunocontraception would be more effective than removing individuals with high mean kinships in preserving the long-term health and viability of the herd.

KEY WORDS Assateague Island National Seashore, *Equus caballus*, fecal DNA, microsatellites, pedigree analysis, wildlife management.

The horses (*Equus caballus*) of Assateague Island National Seashore (ASIS) are one of the few free-ranging animal populations that have been the subject of long-term studies. For >30 years, this population has contributed to our knowledge of feral horse behavior (Keiper 1977, Keiper and Berger 1982, Keiper and Sambraus 1986, Powell 2008), ecology (Zervanos and Keiper 1979, Rutberg and Keiper 1993, Powell et al. 2006), and social structure (Keiper 1978, Rutberg and Greenberg 1990). It has also acted as a natural laboratory for the development and testing of new methods of remote pregnancy testing and immunocontraception (Kirkpatrick et al. 1990, 1993; Kirkpatrick and Turner 1991), as well as for understanding the longer term effects of these technologies on wild mammal populations (Kirkpatrick et al. 1997; Powell 1999, 2000; Powell and Monfort 2001; Kirkpatrick and Frank 2005).

When the National Park Service (NPS) acquired ownership of the horses in 1968, the herd numbered 28. Since then, the population has expanded dramatically, reaching a maximum of 175 horses in 2001. This increase in the horse population has resulted in negative effects on other natural resources. Studies have found that the horses significantly influence the distribution and abundance of native plant species and can affect the dynamics of plant community composition (Furbish and Albano 1994, Zervanos and Keiper 1998). Overgrazing has been shown to impact the physical integrity of sensitive dune features and marshlands through trampling and elimination of soil-binding vegetation (Seliskar 2003, De Stoppelaire et al. 2004).

The NPS recognizes the need to manage the horse population in ways that will provide for the long-term health and viability of the herd as well as minimize adverse impacts on other park resources (U.S. Department of the Interior NPS 1982). Since 1994 the population growth rate has been controlled by a program of immunocontraception for females. Under this program, Porcine Zona Pellucida (PZP) vaccine has been administered annually via a 1.0-cm^3

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dart delivered with a Pneu-Dart (Penu-Dart, Inc., Williamsport, PA) capture gun (Turner et al. 2001, Turner and Kirkpatrick 2002). In 1994 all females that were not part of the 1988–1994 test program were given an initial dose of PZP, which provided antigen recognition in preparation for subsequent management-level application of PZP (Turner and Kirkpatrick 2002). The initial plan was to inoculate all females ages 2–4 years old, then allow them to produce 3 offspring (or a second generation), after which the females would be inoculated annually for the remainder of their lives. In 1998 the plan was amended to allow only 2 foals, and in 2000, it was amended again to allow only 1 foal (Kirkpatrick and Turner 2007). An unexpected result of this program was that the lifespan of females increased substantially (Turner and Kirkpatrick 2002). Although herd size declined from 175 to about 130 in 2009, there is concern that this strategy for population size reduction may have negative impacts on the demography and genetic health of the herd.

Between 1968 and 1990, management of the population was documented primarily by research studies and occasional censuses. It is likely that prior to the beginning of Keiper's study of the horses in 1975, there was occasional straying of stock between the ASIS and Chincoteague National Wildlife Refuge (CNWR) populations, but these events were not well-documented. In 1990 the NPS began a long-term monitoring program that included individual identification and population censuses including group associations, home ranges, and life events. From those data, it was possible to construct maternal pedigrees. Although the presence of males with the female bands may suggest that those males fathered young born to the females, actual matings were rarely observed. Thus, the paternal side of the pedigree was largely unknown.

We used molecular genetic tools and analyses to verify and amend maternal pedigrees. We then used both genetic information and NPS records of historic ranges to infer paternity and construct pedigrees to the extent possible for 154 of the 175 horses living in 2005. We combined these data with historical data in a studbook (a database containing pedigree and life-history information for 500 of the 510 individuals that lived on the island between the mid-1970s when data were first collected and Mar 2006), which we then analyzed using tools designed for population management (Ballou et al. 2010). Thus, we combined molecular genetic analyses, demographic analyses, pedigree analyses, and models developed for captive populations to assess the genetic and demographic status of this wild horse population as well as to predict the effects of efforts to reduce population size.

Recently, a number of papers have addressed the use of pedigrees in the study of wild populations (Haig and Ballou 2002, Herbinger et al. 2006, Berger-Wolf et al. 2007, Pemberton 2008, Van Horn et al. 2008). This approach provides valuable data for the study of ecological and evolutionary processes, including mating systems, inheritance patterns of important traits, and behavior. It also has promise in the area of conservation biology, where information about the relationships between individuals may provide managers with tools for effective management. Our study demonstrates both the power and the challenges of constructing pedigrees for wild populations and using them for conservation management.

STUDY AREA

We collected samples between January and September, 2005, from all 175 living horses at Assateague Island National Seashore, Berlin, Maryland, USA. Assateague Island is a 60-km barrier island off the coast of Virginia and Maryland. The Virginia portion of the island contained the CNWR, which was inhabited by a herd of horses owned and managed by the Chincoteague Volunteer Fire Department. We studied the Maryland herd, which inhabited ASIS and Assateague State Park, managed by the National Park Service and Maryland Department of Natural Resources, respectively.

METHODS

One hundred seventy-six horses (175 living, 1 deceased) were sampled by NPS personnel and volunteers. Each horse was identified using previously recorded coloration, unique physical characteristics such as coat patterns, and herd affinities. We collected an initial set of 25 matched tissue (collected using biopsy darts) and fecal samples to select, optimize, and verify results of candidate genetic markers. We then collected fecal samples from the remaining 150 living horses. Despite repeated attempts, some fecal samples did not yield amplifiable DNA. We resampled 7 of those horses using biopsy darts and collected new fecal samples ($n = 15$) for the others. When DNA sequences revealed that one of the presumed maternal pedigrees (T5) included individuals with different mitochondrial DNA (mtDNA) haplotypes, NPS personnel located a bone from T5, which we used to obtain her DNA to establish which haplotype correctly characterized the T5 lineage.

There were 5 instances in which we found horses to have haplotypes inconsistent with their presumed matrilineal ancestry, which could be explained in several ways: 1) in the field, a fecal sample from one horse could have been mistaken for that of another horse within the same band, or 2) the presumed pedigree was in error. To rule out the former, we collected a second set of samples for all horses involved in these discrepancies.

Genetic Methods

We extracted total genomic DNA from all skin biopsy samples using the tissue protocol of the QIAGEN DNeasy™ DNA extraction kit (Qiagen Inc., Valencia, CA). We extracted DNA from fecal samples using the Guanadine Thiocyanate/Silica (GuSCN) method of Eggert et al. (2005). For the T5 bone sample, we used an autoclaved saw blade and cut the bone in 2 places to obtain material from the area where the marrow would be found. We extracted DNA from this material using the GuSCN method. We performed all extractions from fecal samples and bone in a separate room dedicated to extracting DNA from noninvasively collected samples. We kept this area free

Table 1. Redesigned primers for 4 of the microsatellite loci used to genotype Assateague Island National Seashore (MD, USA) horses sampled in 2005. Sizes are shown in base pairs of DNA sequence (bp).

Locus	Original size (bp)	New size (bp)	Annealing temp (° C)	Primer sequences
ASB2	180	112	55	F: ACTCTTTGGGATCTCCTTCC R: GACTCTTAGCTAATGTTCCCAATG
HMS3	169	130	60	F: TGTGATTCATAAAGGGGATGG R: TTGTCACATAACAAGAAAACAGCTT
HMS6	169	118	59	F: TTCTTCAAATCAGAAACCCATATAGA R: CCCAGCTCCATCTTGTGAAG
HMS7	182	116	60	F: CCCAGACGTGACAAAATTGC R: CCATCCACCAATCTTATTTGG

of polymerase chain reaction (PCR) products and used dedicated materials and equipment.

To amplify mtDNA, we used the primers described by Vilà et al. (2001) to amplify 355 base pairs (bp) of the left domain of the highly variable mtDNA control region. For most samples, we used the first (forward) primer of pair 1 with the second (reverse) primer from pair 3 to amplify the entire 355-bp fragment. For the few samples in which we were unable to obtain enough product for sequencing using these primers, as well as for the T5 bone extract, we used all 3 overlapping primer pairs. We performed amplifications in 25-µl volumes containing 2 µL extracted DNA, 2.5 µL 10× PCR Gold buffer (Applied Biosystems, Foster City, CA), 0.5 µM forward and reverse primers, 2.5 mM dinucleotide triphosphate (dNTP) mix, 1.5 mM $MgCl_2$, 2 µL bovine serum albumin (BSA) and 0.5 u Amplitaq Gold DNA polymerase (Applied Biosystems). The PCR profile consisted of a 10-minute incubation at 95° C followed by 40 cycles of 95° C for 1 minute, 55° C for 1 minute, and 72° C for 1 minute in a PTC100 thermocycler (MJ Research, Waltham, MA). We obtained sequences of the amplification products using the ABI Big Dye ready reaction kit (Applied Biosystems), and detected in a capillary DNA sequencer (ABI 3100; Applied Biosystems).

For microsatellite DNA genotyping, we initially used the StockMarks® for Horses Equine Genotyping Kit (Applied Biosystems) to screen the matched tissue and fecal samples at 11 microsatellite loci. Of these, we found 4 loci to be both reliable and informative (VHL20, HTG4, HTG6, and HTG7). Four others (ASB2, HMS3, HMS6, HMS7) were informative in tissue samples but had high rates of allelic dropout in fecal samples. When amplifying DNA extracted from feces, the size of the microsatellite locus has been found to have a direct effect on amplification rate (i.e., the larger the locus the lower the amplification rates and reliability; Kohn et al. 1995). Therefore, we used the published sequences of these 4 loci to design new primers that amplified less of the flanking region along with the tandem repeat region. Our redesigned primers amplified fragments that were 39–68 bp shorter than the published fragment size of those loci (Table 1). All alleles detected using the original primer set were also detected using the redesigned primers.

We performed microsatellite amplifications in 10-µL volumes containing 1 µL of the extracted DNA, 1 µl 10× PCR Gold buffer (Applied Biosystems), 0.5 µM forward and reverse primers, 2.5 mM dNTP mix, 1.5 mM $MgCl_2$, 1 µL BSA, and 0.5 u Amplitaq Gold DNA polymerase (Applied Biosystems). The profile consisted of a 10-minute incubation at 95° C followed by 40 cycles of 95° C for 45 seconds, 55° C for 35 seconds, and 72° C for 35 seconds in an MJ Research PTC100 thermocycler. We performed genotyping on 2 extractions/sample and scored results after comparison with the sizes of the alleles of the International Society for Animal Genetics–approved control sample from the StockMarks® kit. We calibrated 2 Assateague horse DNAs obtained from biopsy tissues against the control sample and thereafter used them as size controls in all genotyping reactions, along with a negative control to detect contamination of the PCR. We determined allele sizes in an automated sequencer (ABI 3100, Applied Biosystems) using fluorescently labeled forward primers and GeneScan technology (Applied Biosystems). We confirmed all heterozygous genotypes in ≥2 independent PCRs and confirmed homozygotes in ≥3 independent PCRs prior to addition to the final data set.

We aligned sequences of the mtDNA control region and determined haplotypes using SEQUENCHER™ 4.5 (GeneCodes Corp., Ann Arbor, MI). To compare our values of mtDNA diversity with those from Vilà et al. (2001), which included approximately 20 individuals for each established breed, we randomly selected 20 Assateague horses.

We analyzed microsatellite results using GENEPOP (Raymond and Rousset 1995) to determine the number of alleles per locus and the observed and expected heterozygosity values, to test for deviations from expectations under Hardy–Weinberg equilibrium, and to test for linkage disequilibrium. To compare allelic diversity results across lineages with different numbers of individuals, and to compare the Assateague results with those generated for worldwide breeds by Vilà et al. (2001), we estimated allelic diversity for a sample size of 20 using rarefaction in HP-RARE (Kalinowski 2005).

We used the program PROB-ID5 (Waits et al. 2001) to compute $P(ID)_{random}$, the power to differentiate between any 2 randomly chosen individuals, as well as a more conservative measure, $P(ID)_{sib}$, the power to differentiate between siblings. We used results of the more conservative test to determine the power of the 8 loci we used to differentiate between potential sires.

Pedigree Construction and Analysis

We tested presumed maternal pedigrees based on direct observations using genetic data. When we found inconsistencies, we used both genetic data and NPS records regarding band membership and historic ranges to resolve them. Once maternal pedigrees were established, we performed paternity analyses using the genetic data in the program PARENTE (Cercueil et al. 2002) with the following parameters: age difference between sire and young = 3 years, maximum incompatible alleles = 1, maximum missing alleles = 6 (3 loci), error rate = 0.01. Output of this program included all potential sires, number of common alleles and incompatibilities, and probability of paternity for each.

We compared the results of paternity analyses with NPS records to confirm that the males involved were sexually mature and were in the vicinity of the female at the approximate time of conception. When the molecular genetic analyses were unable to identify a sire for an individual, we determined whether the most likely sire had died and was, thus, unavailable for sampling in the survey. In the few cases where the program suggested that several sires were equally probable, we used NPS records of historic ranges to narrow the field to only males that were known to have been in the proximity of the female. We included in the pedigree sires that died or were determined using range data only when NPS records were unambiguous. We did not include in the demographic analyses 3 living individuals that were transferred out of the population in the early to mid-1990s, but used their data in genetic calculations if they left descendants on the island.

Although our data did not allow us to trace lineages of the current population back to the original horses acquired by the NPS in 1968, we were able to trace many of the lineages back to individuals from the population as first fully described by Keiper (1985) or to their first-generation descendants. Without additional data on the history of these individuals, we assumed that they were unrelated and listed their parents as unknown. Thus, all analyses from the studbook data (the level of inbreeding, retention of genetic diversity) are relative to these horses, which we refer to as the founders. Although treating each of these individuals as unrelated is most likely erroneous, pedigree analysis only assumes that they are equally related amongst themselves and that any further accumulation of inbreeding or loss of genetic diversity is relative to that of the founders. Recent research has shown that, in the long term, the cost of not knowing founder relationships when using mean kinship measures is generally low (Rudnick and Lacy 2008).

We used the Single Population Animal Records Keeping System (SPARKS v. 1.52; International Species Information, Eagen, MN) to generate a studbook of the population, containing parentage data for every living and deceased member as well as basic biological data such as age, sex, and reproductive history. We then analyzed the pedigree and life-history data in PM2000 (<http://www.vortex9.org/pm2000.html>, accessed 17 Jun 2008; Pollak et al. 2002) to perform demographic analyses and make predictions about the effects of management actions on the demography and genetics of the Assateague horses. We evaluated the current age structure, the contributions of the individual founders to the genetic diversity of today's herd, changes in levels of inbreeding over time, the rate of loss of genetic diversity over the past several generations, and the overall levels of kinship among animals in the current population. We calculated overall levels of kinship as the average mean kinship (MK), which is the average of the pair-wise kinships of all pairs living in the population.

We then used the MK measure to evaluate the relatedness of each individual to the population as a whole (Ballou and Lacy 1995). Individuals with low MK are distantly related to other individuals and may represent an important component of the genetic legacy of the founders that is not present in other individuals. Mean kinship has outperformed other measures in terms of heterozygosity and allelic diversity retention and inbreeding minimization, as shown theoretically through simulations (Ballou and Lacy 1995, Fernandez and Toro 1999, Toro et al. 1999, Toro 2000) and by experimental evidence in fruit flies (*Drosophila* spp.; Montgomery et al. 1997). Although MK values are most often used to identify the genetically valuable animals in the population, they can also be used to identify those individuals whose genes are overrepresented. To determine how removing specific individuals might affect genetic diversity, we calculated MK values for each horse using PM2000. We removed the individual with the highest MK, after which we recalculated MKs for all remaining individuals. We repeated this process until either 25 or 50 individuals were removed. We did not include in the calculation 4 individuals whose parentage was completely unknown.

Table 2. Population-level genetic diversity measures for Assateague Island National Seashore (MD, USA) horses in 2005. Loci for which heterozygosity (H) values deviate significantly from expectations under Hardy–Weinberg equilibrium are indicated by *.

Locus	No. alleles	H obs	H exp
ASB2R	10	0.759	0.825
HTG4	5	0.682	0.741
HTG6*	5	0.861	0.653
HTG7	8	0.514	0.541
HMS3R	9	0.762	0.713
HMS6R	7	0.673	0.730
HMS7R	8	0.645	0.625
VHL20	7	0.738	0.732
Average	7.4	0.704	0.695
SD	1.8	0.102	0.086

RESULTS

Genetic Analyses

We detected 3 mtDNA haplotypes, defined by 12 12-polymorphic bases in the sequence. Haplotype A differed from Haplotype B at 7 positions, A differed from C at 7 positions, and B differed from C at 10 positions (GenBank accession no. GU014400–GU014402). The most common haplotype was A (N = 124, 71%), whereas B and C were less common (B = 20%, C = 9%). In the random sample of

Table 3. Measures of genetic diversity within maternal lineages of Assateague Island National Seashore (MD, USA) horses in 2005. Uncorrected allelic diversity (A) and allelic diversity estimated using rarefaction for a sample size of 4 (A[HP-RARE]) are shown along with observed and expected heterozogosity (H) values.

Lineage	*N*	A	A (HP-RARE)	H obs	H exp
M2	10	4.1	2.5	0.83	0.66
M6	8	3.0	2.2	0.60	0.55
M17	5	3.0	2.2	0.62	0.58
N2	12	3.4	2.3	0.74	0.64
N6	29	5.0	2.5	0.71	0.69
N9	35	6.0	2.7	0.72	0.72
N10	9	4.0	2.5	0.68	0.65
T3	14	4.0	2.3	0.65	0.63
T5	27	5.1	2.6	0.70	0.69
T6	14	4.0	2.6	0.68	0.63
X16	4	2.3	2.1	0.54	0.44
Single horse lineages	8	3.9	2.5	0.75	0.69
Average		4.0	2.4	0.69	0.63
SD		1.0	0.2	0.08	0.08

20 horses we selected to compare with diversity values from Vilà et al. (2001), we found all 3 mtDNA haplotypes and a nucleotide diversity value of 0.011 (±0.006 SD).

Of the 176 horses, we successfully genotyped 116 at all 8 loci, 24 at 7 loci, 20 at 6 loci, and 12 at 5 loci. In total, we successfully genotyped 98% of samples and only 4 samples could not be genotyped at enough loci to be included in these analyses. We computed probability of identity values using the initial set of 25 biopsy samples. The $P(ID)_{random}$ for all 8 loci was 0.00005 and the $P(ID)_{siblings}$ was 0.002; for 6 loci they were $P(ID)_{random} = 0.00002$ and $P(ID)_{siblings} = 0.009$; and for 5 loci values were $P(ID)_{random} = 0.0002$ and $P(ID)_{siblings} = 0.023$. Because we genotyped all but 12 horses at >5 loci, these results indicate that our panel of loci had sufficient power to perform pedigree and paternity analyses.

With one exception, all loci conformed to Hardy–Weinberg expectations (Table 2). Locus HTG6 had an excess of heterozygotes, a surprising result because most studies using fecal DNA suffer from problems of allelic drop-out (Gagneux et al. 1997). Because the slight excess of heterozygotes did not affect our ability to assess the genetic variability of the population and enhanced our ability to assess parentage, we retained this locus in the analyses. We found an average of 7.4 (±1.8 SD) alleles/locus and average heterozygosity of 0.704 (±0.102 SD). Rarefaction methods for a sample size of 20 (Kalinowski 2005) rendered a value of 4.4 (±0.9 SD) alleles/locus.

Once we verified and corrected the presumed maternal pedigrees, we assessed levels of diversity for each of the resulting 11 maternal lineages. Each lineage presented comparable allelic diversity (Table 3). When we estimated allelic diversity for a sample size of 4 (no. of individuals in the X16 lineage) in HP-RARE, allelic diversity values ranged from 2.1 to 2.7. Heterozygosity values were comparable among lineages, with the exception of X16, which appeared to have less genetic diversity (Table 3). Although C is the rarest mtDNA haplotype, horses of that lineage carry few rare (frequency ≤ 0.05) microsatellite alleles (Table 4).

Pedigree Construction and Analysis

When we compared mtDNA haplotypes and microsatellite genotypes with the presumed maternal pedigrees, there were 5 instances in which horses had mitochondrial haplotypes or microsatellite genotypes inconsistent with their presumed matriline. After sequencing and genotyping a new sample from each of these horses, we found 2 instances in which the original fecal sample represented another member of the band, rather than the intended individual, and 3 instances in which the presumed matrilineal relationships were incorrect. In those 3 cases, we used genetic data and NPS records of band ranges and compositions to resolve the relationships and amend the maternal pedigrees. Thus, we confirmed presumed maternal pedigrees for 169 of the 172 genotyped horses, and corrected the remaining 3.

Paternity analyses in PARENTE identified sires for 55 individuals. Of these, 36 were the presumed sire (the band M), 3 resolved uncertainties that resulted from the female changing bands around the time of conception, 7 were subordinate males in the presumed sire's band, and 9 were instances in which sires were not in the same band. For the remaining 120 horses, examination of NPS records revealed that the most likely sires died before the survey. Using those records, we were able to infer the probable sire for 99 of these individuals. We were unable to determine the sire for the remaining 21, which were largely the first generation offspring of the founders.

Table 4. Distribution of rare (frequency [freq] ≤ 0.05) microsatellite alleles in Assateague Island National Seashore (MD, USA) horses in 2005. We show total number of individuals, mitochondrial DNA (mtDNA) haplotype, and number of individuals with each of the rare alleles for each lineage.

			Lineage and mtDNA haplotype										
Locus	Allele	Freq	A, M2	B, M6	A, M17	A, N2	A, N6	A, N9	A, N10	A, T3	B, T5	C, T6	A, X16
VHL20	98	0.01						1	1		1		
HMS6R	106	0.04					6	5	2		1		
HTG7	122	0.01	1										
HTG7	124	0.01						1	1		1		
HMS7R	106	0.04		1			3	6			3	2	
HMS7R	110	0.02		1				2		1	2		
HMS7R	114	0.01			1			1					
HMS7R	110	0.02					1	4					
N			10	8	5	12	29	35	9	14	27	14	4

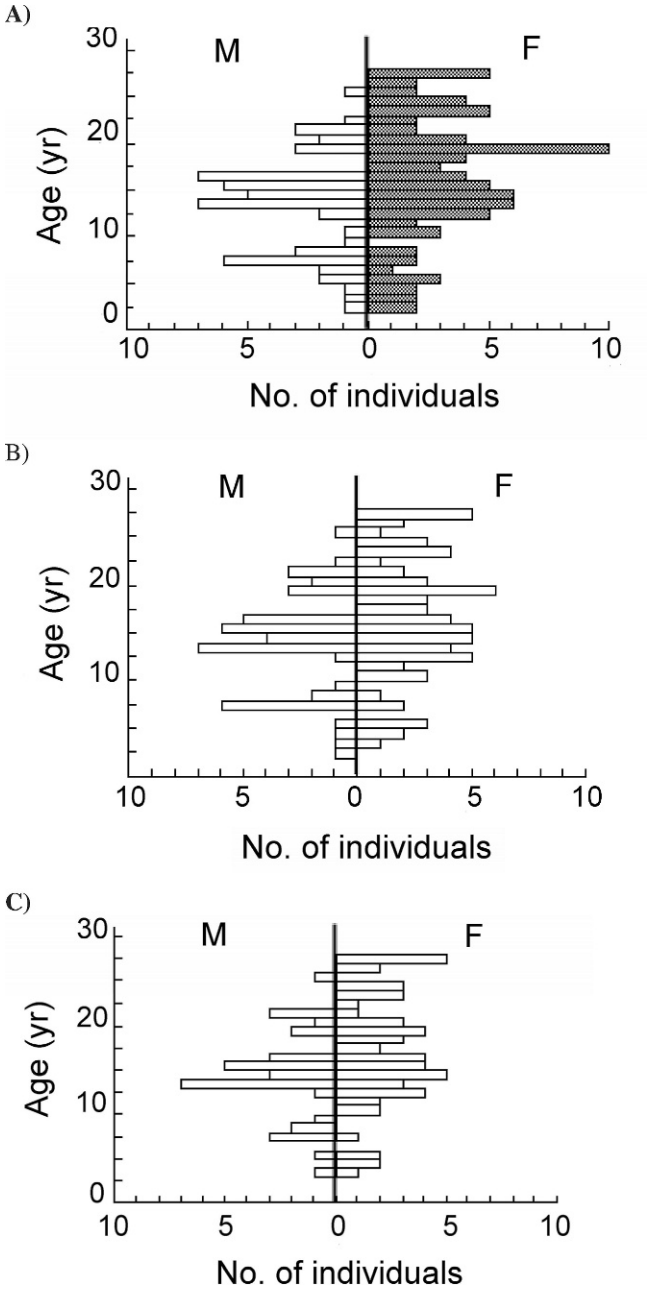

Figure 1. Demography and age structure of the Assateague Island National Seashore (MD, USA) horse population. (A) living population in 2005; (B) after removing 25 individuals based on mean kinship values; (C) after removing 50 individuals based on mean kinship values.

We then combined the pedigree with historical data to create the studbook. The full studbook consisted of data for 510 horses from the period when Keiper started collecting detailed individual animal data in the mid-1970s through March 2006. Of the 510, all but 10 had enough data to enter into the SPARKS database.

Analyses Using the Pedigree

The sex ratio of the extant population was strongly female-biased. Inspection of the age pyramid (Fig. 1A) revealed 3 age cohorts (0–9 yr, 10–15 yr, and ≥16 yr). In males, 31.6% were in the youngest cohort, 49.1% were intermediate, and

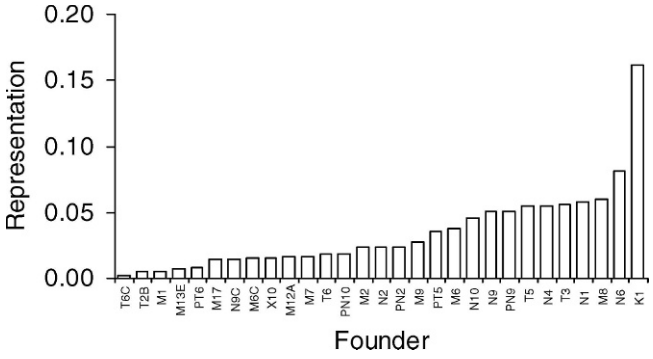

Figure 2. Founder representation in the 2005 Assateague Island National Seashore (MD, USA) horse population. Individuals that do not appear in the studbook but were unrecorded mates of individuals in the studbook are designated by a P prefix.

19.3% were in the oldest cohort. The female population contained many older individuals, with 21.9% in the youngest cohort, 30.2% in the intermediate cohort, and 47.9% in the oldest cohort.

The current population can be traced back to 39 founders (Fig. 2). Because we could not identify sires for some horses, we could trace ancestry to these individuals for only 70% of the living population (Ballou et al. 2008). Representation in this analysis refers to the proportion of the current gene pool that was contributed by each founder. Regardless of their provenance, most of the founders contributed relatively equally, with the exception of K1 (studbook no.178), a very successful breeding male that continued to sire offspring until his death in 1992.

In 2006 there were 144 living horses, 21 of which we found to be inbred, with inbreeding coefficients ranging from 0.0156 to 0.2813 (e.g., Horse N6ELSC; Figs. 3, 4). Average inbreeding in the population was 0.0206. Historically, inbreeding accumulated in the population at the rate of approximately 1%/year (Fig. 5). We calculated the inbreeding effective size of the population over the last several years using

$$1-F_t=\left(1-\frac{1}{2N_e}\right)^t$$

where F_t is the average inbreeding at generation t and N_e is the inbreeding effective size. We simplified this to (Frankham et al. 2002)

$$\ln(1-F_t)\approx -t\left(\frac{1}{2N_e}\right)$$

$$\text{and } N_e\approx\frac{-t}{2\ln(1-F_t)}.$$

Using F_t = 0.0206 and t = 1.8 generations (assuming generation length was approx. 10 yr), then $N_e \approx 48$, or approximately 33% of the population census.

We derived an unbiased estimate of the loss of genetic diversity since the founder population of the mid-1980s by conducting pedigree analysis of only that portion of the pedigree that is known (Ballou and Lacy 1995). Analysis of the pedigree rather than the molecular data is preferable

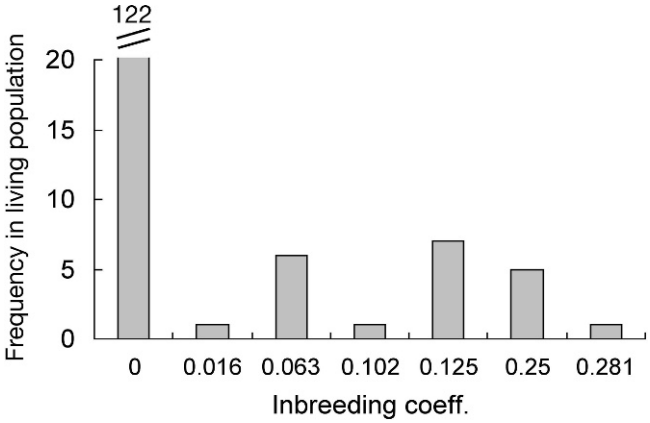

Figure 3. Distribution of estimated inbreeding coefficient in the 2005 Assateague Island National Seashore (MD, USA) horse population.

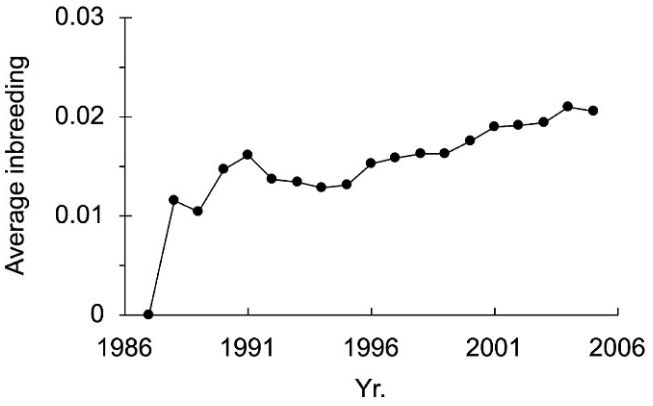

Figure 5. Increase in average inbreeding in the Assateague Island National Seashore (MD, USA) horse population.

because the molecular analysis does not include genotypes back to the 1980s; founder population and pedigree analysis provide an estimate of genome-wide changes in diversity (Fernandez et al. 2005). Analysis of the pedigree using PM2000 indicated that average MK of the population was 0.042. We estimated the retained genetic diversity (GD) as

$$\mathrm{GD} = 1 - \text{average (MK)}$$

Thus, the current population has retained 95.8% of the genetic diversity of the population from which it was derived (i.e., the population 2 horse-generations ago). The loss of 4.2% of diversity over this time equates to an eigenvalue effective population size (Templeton and Read 1994) of 23, or approximately 16% of the census number. We estimated the future loss of genetic diversity using

$$\mathrm{GD}_t = \mathrm{GD}_{(t-1)} \times (1 - 1/2N_e)$$

where GD_t is the proportional retention of genetic diversity at generation t and N_e is the effective population size (Kimura and Crow 1963). Projecting this rate of loss into the future, we found that within 5 generations (approx. 50–60 yr), the Assateague herd will be expected to retain 86% of the diversity of the founders.

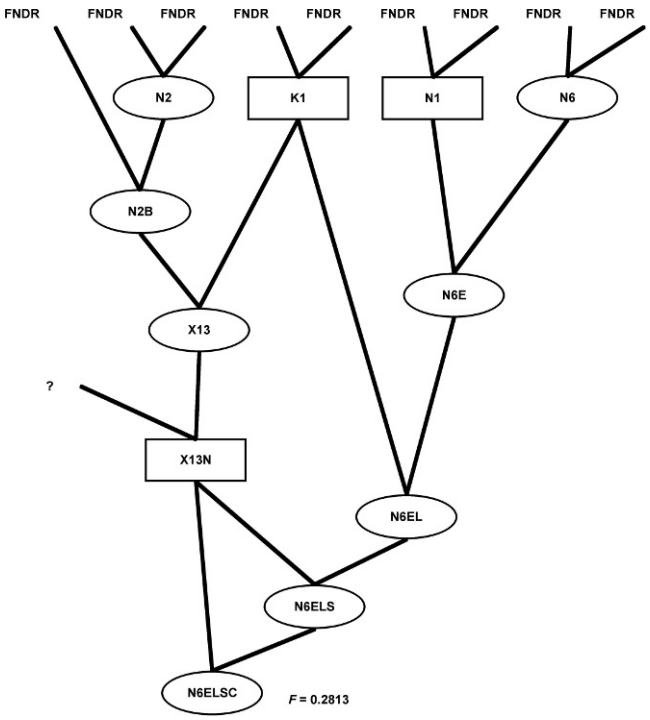

Figure 4. Pedigree of Assateague Island National Seashore (MD, USA) horse N6ELSC; circles are females, squares are males. The designation FNDR represents either a founder or as far back as we could trace the pedigree; ? indicates an unknown sire.

Genetic Management Through Selective Removal

Because gene diversity and average mean kinship are directly related, results of simulations of the genetic effects of selective removal of horses based on MK confirm that removal of animals with high MK values increases estimates of retained genetic diversity in the herd by reducing average MK (Table 5). The magnitude of these changes on the current population increases as more animals are removed.

The demographic effects of removals based on MK show that removing the 25 horses with the highest MK affected all age cohorts but most strongly affected young horses ($n_{\mathrm{males}} = 5$ and $n_{\mathrm{females}} = 5$) and older females ($n = 11$; Fig. 1B). Removal of 25 additional horses affected mostly older horses ($n_{\mathrm{males}} = 7$ and $n_{\mathrm{females}} = 11$; Fig. 1C). Under both scenarios, approximately one-third of the population would continue to be made up of females in the oldest cohort.

Table 5. Predicted effects of selective removal of horses with high mean kinship on the genetic diversity of the Assateague Island National Seashore (MD, USA) horse population.

Parameter	2005 population	25 removed	50 removed
Retained diversity of founders	0.958	0.964	0.968
Average mean kinship	0.042	0.036	0.032

DISCUSSION

There are only a few populations around the world that have been studied in as much detail and over a comparable period of time as the Assateague horses. They include the red deer (*Cervus elephas*) of the Isle of Rhum, Scotland (Clutton-Brock et al. 1982), the Soay sheep (*Ovis aries*) of the St. Kilda archipelago, Scotland (Clutton-Brock and Pemberton 2004), the elephants (*Loxodonta africana*) of Amboseli National Park, Kenya (Moss 2001), and the chimpanzees (*Pan troglodytes*) of Gombe National Park, Tanzania (Lodwick et al. 2004). These populations provide unparalleled windows into behavioral ecology, reproduction, population dynamics, and effects of natural selection, none of which can be studied in the short-term. The pedigrees we developed will make the Assateague horses an even more valuable resource for the study of the ecology and management of large mammal populations.

The available evidence suggests that the ASIS horse population has been largely isolated for nearly 40 years. We detected only 3 unique mtDNA haplotypes, providing support for the notion that there has been little migration into the population. In their study of established breeds, Vilà et al. (2001) documented, on average, 7.4 haplotypes/breed (range = 3–9) and found that mtDNA nucleotide diversity averaged 0.022 (range = 0.012–0.027). Those values are comparable to the mtDNA diversity found in large wild ungulate populations (Arctander et al. 1996). In our random sample of 20 horses, we found all 3 mtDNA haplotypes and a nucleotide diversity value of 0.011 (±0.006 SD). Thus, levels of mtDNA diversity we observed in the Assateague herd are low in comparison to those found in established breeds and other wild ungulate populations, most probably as a result of isolation.

Despite having reduced diversity at mtDNA, the horses have retained high levels of nuclear genetic diversity. Goodloe et al. (1991) found that diversity levels at allozyme loci in horses from Assateague Island were comparable to those found in larger mainland populations. Their study, however, sampled only the CNWR herd on the Virginia end of the island, which has been managed independently and separated from the ASIS herd by a fence. The CNWR herd has been subject to frequent introductions of individuals of other breeds, whereas the ASIS population has been isolated from the CNWR herd for 40 years and has had no documented introductions. Our results indicate that the levels of allelic diversity and heterozygosity of ASIS horses were indeed comparable to mainland populations and to those found by Vilà et al. (2001) for established breeds.

In light of the reduced mtDNA haplotype diversity, the relatively high level of nuclear diversity is interesting. In St. Kilda's Soay sheep population, heterozygosity levels were higher than expected because inbred individuals that suffered higher parasite loads had higher mortality rates under the harsh environmental conditions (Coltman et al. 1999). Future monitoring of the Assateague horses may reveal that a similar process has been operating here. Alternatively, the managed breeding programs of mainland horses may result in inbreeding levels similar to those found in the isolated Assateague population.

One factor that may have contributed to high levels of nuclear genetic diversity is that the effective size of the horse population has been about 20–30% of the total size over the past 2–3 decades. This effective population size is substantially higher than the 10–11% average found for other wildlife species (Frankham 1995) but is in line with effective sizes typically found in captive populations (Ballou et al. 2010). The elevated effective size may result from the immunocontraception program, which tends to reduce variance in family sizes by limiting the number of offspring a female can have.

Another factor may be the approximately equal founder representation. Managers of captive species have found that equalizing the contribution of founders is an effective way to maintain genetic diversity (Haig et al. 1990). Feral horses have a harem system, in which one male defends a group of females and fathers their young. This social system is not expected to be conducive to equal genetic representation of males in the next generation. However, the ASIS horse population started out small and grew rapidly from 1968 to 1994. It is likely that there were many opportunities for breeding as the population expanded, as well as available habitat for formation of new harems.

In recent years, that situation has changed somewhat. There have been 30 bands on the island for at least the last decade, but the number of bands is now dropping along with population size. Although this might suggest that opportunities for breeding arise today primarily through the deaths of breeding males, it appears that young bachelor males have many more breeding opportunities than would be expected based only on death rates of current harem males. We detected 7 instances in which a subordinate male in the harem male's band sired offspring and 9 instances in which males outside the harem sired young. In fact, it is not unusual for young bachelor males to take over full bands or parts of bands (A. Turner, NPS, personal communication). Nevertheless, the presence of many individuals in the youngest age cohort with higher MK values suggests that inbreeding levels are increasing in the population.

We estimate the effective size of this population at 48 for the inbreeding effective size and 23 for the effective size associated with the loss of genetic diversity (eigenvalue effective size), which, respectively were 33% and 16% of the census number. The lower ratio is similar to the average N_e/N ratio of 11% found by Frankham (1995) in his review of effective sizes in wildlife populations. The higher N_e/N ratio for the inbreeding effective size may indicate an avoidance of inbreeding. We based the ratio, however, on the assumption that unknown animals were unrelated to known animals, which is not likely to be correct. Thus, this estimate should be considered an upper bound of N_e/N for this population.

Our results suggest that if maintenance of the genetic diversity that may allow a population to respond to future environmental and ecological challenges is one of the goals of the management program, managing the population to maintain rare mtDNA haplotypes is not likely to be a

successful strategy. Although mtDNA haplotype C is the rarest in this population, horses in that lineage do not harbor rare microsatellite alleles. Microsatellite loci, especially dinucleotide loci, are generally found in noncoding regions of the genome and are thus unlikely to affect the fitness of the individual. However, they may be viewed as proxies for the genome as a whole, making the assumption that lineages that contain many rare microsatellite alleles may also harbor rare alleles at loci that are likely to affect fitness.

Construction of maternal pedigrees was aided by the fact that many of the dams were alive and sampled in the survey, which was not true for the sires. The presence of these dams is likely a result of the immunocontraception program. Release from the stresses of pregnancy and lactation has resulted in an increased lifespan of 5–10 years for females, which has extended the amount of time needed to reduce population size using contraception alone (Turner and Kirkpatrick 2002, Kirkpatrick and Turner 2007). With only limited breeding, the population now has comparatively fewer individuals in the younger age classes. The decreased number of breeding-age females may put the population at greater genetic and demographic risk in the long term, particularly if some unknown event led to an increase in mortality of females.

MANAGEMENT IMPLICATIONS

Genetic management in the form of selective removal of horses with high MK values would decrease average kinship in the population immediately. However, it would also decrease the number of young individuals available for breeding, limiting future reproductive potential. There are few older females with high MK. Removing all or most of these individuals may disrupt the social structure of the bands, and older horses may adapt less easily to adoption. Our results suggest that a combined strategy of controlled breeding and immunocontraception would be more effective in preserving the long-term health and viability of the herd. By 2009 the immunocontraception program reduced the population size to about 130, and indications are that it will continue to decline. Once the population has reached a number at which it is no longer having severe negative impacts on the island ecosystem, not only will breeding need to resume to preserve the demographic integrity of the herd, but occasional introductions of horses may need to be done to maintain genetic diversity.

Our study demonstrates the power of using pedigree analyses to provide the data needed for effective management. However, it also demonstrates the inherent challenges. Years of observational data were available from which to construct maternal pedigrees. Discrepancies in the maternal pedigrees of the 175 horses living in 2005 were few and were resolved relatively easily using both the mtDNA and microsatellite markers. Paternity inference was far more difficult, because many of the sires were no longer present, which is likely to be a problem for studies of wild populations, especially those that are not both temporally and spatially closed. Resolving cases in which >1 potential sire was equally probable was aided by NPS records of ages and historic ranges, as well as by knowledge of the inheritance of coat color patterns, data that may be unavailable in studies of wild populations. Thus, although we are optimistic about the potential uses of pedigree data for answering questions in ecology and evolution and providing data for conservation management, we caution that more theoretical and empirical work is needed before such studies will be feasible for most wild populations.

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