

The genealogy and genetic viability of reintroduced Yellowstone grey wolves

BRIDGETT M. VONHOLDT,* DANIEL R. STAHLER,*† DOUGLAS W. SMITH,† DENT A. EARL,* JOHN P. POLLINGER* and ROBERT K. WAYNE*

*University of California, Los Angeles, Ecology and Evolutionary Biology, 621 Charles E. Young Dr South, Los Angeles, CA 90095, USA, †Yellowstone Center for Resources, National Park Service, PO Box 168; Yellowstone National Park, Wyoming 82190, USA

Abstract

The recovery of the grey wolf in Yellowstone National Park is an outstanding example of a successful reintroduction. A general question concerning reintroduction is the degree to which genetic variation has been preserved and the specific behavioural mechanisms that enhance the preservation of genetic diversity and reduce inbreeding. We have analysed 200 Yellowstone wolves, including all 31 founders, for variation in 26 microsatellite loci over the 10-year reintroduction period (1995–2004). The population maintained high levels of variation (1995 $H_0 = 0.69$; 2004 $H_0 = 0.73$) with low levels of inbreeding (1995 $F_{IS} = -0.063$; 2004 $F_{IS} = -0.051$) and throughout, the population expanded rapidly ($N_{1995} = 21$; $N_{2004} = 169$). Pedigree-based effective population size ratios did not vary appreciably over the duration of population expansion (1995 $N_e/N_g = 0.29$; 2000 $N_e/N_g = 0.26$; 2004 $N_e/N_g = 0.33$). We estimated kinship and found only two of 30 natural breeding pairs showed evidence of being related (average $r = -0.026$, SE = 0.03). We reconstructed the genealogy of 200 wolves based on genetic and field data and discovered that they avoid inbreeding through a wide variety of behavioural mechanisms including absolute avoidance of breeding with related pack members, male-biased dispersal to packs where they breed with nonrelatives, and female-biased subordinate breeding. We documented a greater diversity of such population assembly patterns in Yellowstone than previously observed in any other natural wolf population. Inbreeding avoidance is nearly absolute despite the high probability of within-pack inbreeding opportunities and extensive interpack kinship ties between adjacent packs. Simulations showed that the Yellowstone population has levels of genetic variation similar to that of a population managed for high variation and low inbreeding, and greater than that expected for random breeding within packs or across the entire breeding pool. Although short-term losses in variation seem minimal, future projections of the population at carrying capacity suggest significant inbreeding depression will occur without connectivity and migratory exchange with other populations.

Keywords: conservation, genealogy, heterozygosity, inbreeding, viability, wolves

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Introduction

The reintroduction of extirpated species to their native habitats is an important step in the restoration of ecosystem function in human-altered landscapes. However, many reintroductions are actual or functional failures due to poor quality of the recovery habitat, the inexperience of reintro-

duced captive-reared individuals, too few founding individuals, or failure to release individuals into a region of their historic distribution (Griffith *et al.* 1989; Wolf *et al.* 1998; Breitenmoser *et al.* 2001; Frankham *et al.* 2002). Few model reintroductions are available for study to understand how long-term viability can be retained. A key element of successful reintroduction programmes is the integration of genetic management into the scientific design, in addition to an understanding of ecology and demography of the reintroduced species (Wolf *et al.* 1998; Miller *et al.* 1999;

Correspondence: Robert K. Wayne, Fax: 310-825-3987; E-mail: rwayne@ucla.edu

Breitenmoser *et al.* 2001; Frankham *et al.* 2002). Because reintroduced populations typically have small effective population sizes and are isolated, the effect of inbreeding and loss of genetic variation on population viability can be substantial (Wright 1931; Nei *et al.* 1975; Frankham *et al.* 2002). Thus, founding populations should be large and composed of genetically diverse individuals to reduce fitness costs associated with inbreeding depression and to allow for an adaptive response to changing conditions (Lande 1995; Keller & Waller 2002). Factors that influence the genetic structure of the reintroduced population also must be considered so as to maximize the genetic variation retained during the recovery process. For example, mating system and the degree of sociality can influence fine-scale genetic structure through patterns of breeding and population assembly rules (Chesser 1991a, b; Sugg *et al.* 1996; Randall *et al.* 2007). In this regard, population structure is predicted to be largely influenced by sex and kinship bias in dispersal patterns, inbreeding avoidance, and social barriers to gene flow (Sugg *et al.* 1996; Girman *et al.* 1997; Smith *et al.* 1997; Peakall *et al.* 2003). Finally, management should aim to establish and maintain population connectivity, which can greatly influence population growth, gene flow, and genetic variation (Keller & Waller 2002; Riley *et al.* 2006).

With respect to the presence and relative abundance of large carnivores, all natural habitats in the contiguous United States are highly altered from pre-Columbian conditions. Large carnivores often exert a top-down impact on ecosystems with effects on prey and vegetation (Terborgh *et al.* 1999, 2006; Schmitz *et al.* 2000; Smith *et al.* 2003; Soule *et al.* 2003; Hebblewhite *et al.* 2005). The grey wolf (*Canis lupus*) is the only top carnivore to be effectively extirpated from the American West and historic populations had higher genetic variability than elsewhere in North America, suggesting a population of more than 350 000 wolves (Leonard *et al.* 2005). After being absent for approximately 70 years, the grey wolf was reintroduced to Yellowstone National Park (YNP), Wyoming, in 1995 and 1996 as part of wolf restoration to the northern Rocky Mountains (Bangs & Fritts 1996; Phillips & Smith 1996). A founding stock of 31 wild-born individuals from Canada was used to establish a breeding population, with 10 additional wolves translocated from northwestern Montana augmenting the population in 1997 (Bangs *et al.* 1998). Strategic guidelines regarding genetic aspects of recovery included selecting an adequate number of founders from two distinct source populations and reintroducing extant family groups to promote early reproduction and social stability (USFWS 1994; Fritts *et al.* 1997). In the 10 years following their initial release, wolves have recolonized the 8991 km² park and several adjacent portions of the 72 800 km² Greater Yellowstone Area (GYA). The population expanded rapidly due to an abundance of prey and reduced human exploitation, which are both considered key habitat criteria for wolves

(Mech 1970; Fritts *et al.* 2001). This growth represents the products of founder reproduction only, as the isolation of the GYA from other wolf populations prevented any influential immigration (Oakleaf *et al.* 2006; von Holdt unpublished data). Currently, there are over 300 wolves in the GYA (USFWS 2007) which affect many aspects of ecosystem function (Smith *et al.* 2003). The remarkable success of the reintroduction of the wolf to Yellowstone provides a unique opportunity to understand the role of individual breeding patterns and social structure in the preservation of genetic diversity.

Wolves live in territorial social groups whose members cooperate to capture prey, raise young, and defend resources from competitors (Mech 1970; Mech & Boitani 2003b). Wolf packs most commonly represent families consisting of a single breeding pair and their offspring of one or more litters (Murie 1944; Mech 1970). However, packs may also include siblings or previous offspring of one of the breeding pair, and may include nonreproductive individuals unrelated to pack members (Mech & Boitani 2003a). Due to such kinship structuring in wolf populations, levels of genetic heterogeneity will be influenced by aspects of their mating system, such as reproductive skew, inbreeding avoidance, and access to unrelated mates. For example, although it has been shown that inbreeding avoidance is an important constraint on wolf behavioural ecology (Smith *et al.* 1997), some wolf populations have experienced bottlenecks or founding events resulting in genetic deterioration and inbreeding depression (Wayne *et al.* 1991; Peterson *et al.* 1998; Liberg *et al.* 2005). Additionally, the effective population size (N_e) is much smaller than census population size (N_c) in wolf populations, reflecting the limited number of breeders (Nunney 1995; Frankham 1996; Aspi *et al.* 2006). Because N_e determines the rate of loss of genetic variation, inbreeding, and the fixation of deleterious alleles (Wright 1969), understanding changes in N_e are important for long-term genetic viability of wolf populations (Peterson *et al.* 1998; Randi *et al.* 2000; Aspi *et al.* 2006).

In this study, we constructed a pedigree of the Yellowstone population involving all founder individuals and 169 of their descendants over the past decade. Few studies to date have been able to resolve extensive relationships in a wild endangered species (Taylor *et al.* 1997; Kalinowski *et al.* 1999; Ralls & Ballou 2004; Liberg *et al.* 2005). This pedigree, based on field and genetic data, was used to explore trends in genetic diversity, population structure, and effective population size. We describe how reproduction, pack formation and kinship influenced the observed genetic variation, and identify population assembly rules governing the preservation of variation in this rapidly expanding wolf population. Additionally, we evaluated the success of observed breeding behaviour in maintaining genetic variation as compared to simulated breeding

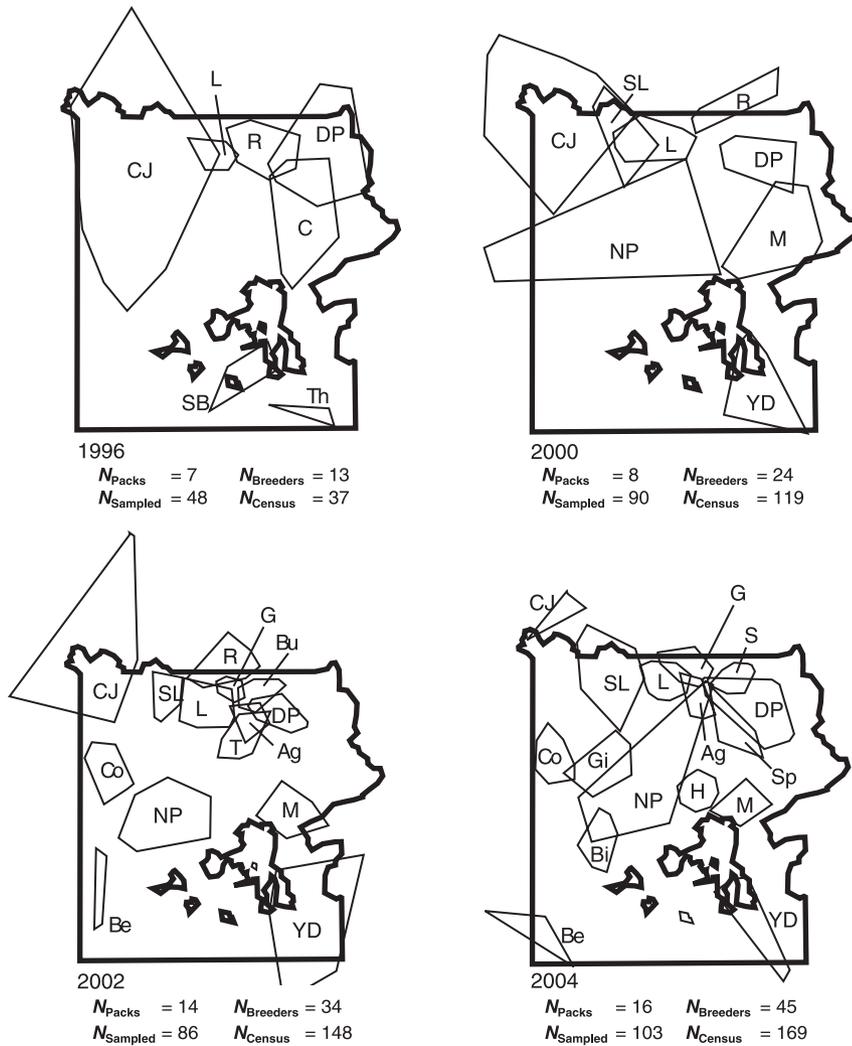


Fig. 1 Pack distributions, number of packs, number of individuals sampled, number of breeding individuals, and census size for Yellowstone National Park wolves (1995–2004). Polygons represent the pack territories. Number of breeders was based on field observations of attempted or actual copulations as well as documented pup production in packs. (Ag, Agate Creek; Be, Bechler; Bi, Biscuit Basin; Bu, Buffalo Fork; CJ, Chief Joseph; Co, Cougar Creek; C, Crystal Creek; D, Druid Peak; G, Geode Creek; Gi, Gibbon Meadows; H, Hayden; L, Leopold; M, Mollie’s; NP, Nez Perce; R, Rose Creek; Sh, Sheep Mountain; S, Slough Creek; SB, Soda Butte; Sp, Specimen Ridge; SL, Swan Lake; Th, Thorofare; T, Tower; and YD, Yellowstone Delta).

scenarios. Finally, we projected changes in genetic variability into the future and discuss management strategies for maintaining variation. This study provides a unique assessment of endangered species recovery, and facilitates a greater understanding of the importance of conservation actions on genetic viability and population persistence.

Materials and methods

Sample collection design

Blood and tissue samples were collected between 1995 and 2004 from 200 wolves from YNP by means of helicopter darting and post-mortality sampling (Fig. 1). Our sampling represents 23 packs: Agate Creek, Bechler, Biscuit Basin, Buffalo Fork, Chief Joseph, Cougar Creek, Crystal Creek, Druid Peak, Geode Creek, Gibbon Meadows, Hayden, Leopold, Lone Star, Mollie’s, Nez Perce, Rose Creek, Sheep Mountain, Slough Creek, Soda Butte, Swan Lake, Thorofare,

Tower, and Yellowstone Delta. All 31 founding Canadian wolves were sampled prior to their release in YNP. Additionally, 10 pups (Sawtooth pack) were translocated from northwestern Montana in 1996 after their parents were killed as part of a livestock depredation control action in 1996 and represented additional individuals unrelated to Yellowstone founders. Only two of these, however, were observed to reproduce in the wild. The proportion of individuals radio-collared during the study period ranged from 25 to 86% of the total Yellowstone census size (N_C ; range 21–174 wolves), defined as all living wolves at the end of the calendar year. All radio-collared individuals were aged and sexed and pack membership, social status, and location of the pack within the park were recorded at least once per week. Most (81%) of the radio-collared wolves were genetically sampled. Changes in pack membership, new pack formation and an individuals’ social status were determined via aerial and ground monitoring of collared and uncollared individuals. Field-based parentage

was used to corroborate genetic analysis, and was based on observed dominant status of males and females, copulatory ties, morphological evidence of pregnancy prior to denning period, and denning behaviour. At dens, the presence of a lactating female indicated maternity; however, multiple breeders at den sites made it difficult to resolve field-estimated parentage in these circumstances without confirming genetic data.

Microsatellite genotyping

DNA was extracted from whole blood, tissue, hair, and serum using the QIAGEN QIAamp DNA Mini kit and the manufacturer's protocol. We genotyped individuals for 30 domestic dog microsatellite loci that were screened for amplification and polymorphic content on a test panel of 24 grey wolf samples: PEZ5, PEZ6, PEZ8, PEZ11, PEZ12, PEZ15, PEZ19 (J. Halverson in Neff *et al.* 1999), FHC2001, FHC2004, FHC2010, FHC2054, FHC2088, FHC2137, FHC2324, FHC2611, FHC2658, FHC2670, FHC2766, FHC2785, FHC2790, FHC2869, FHC2914, FHC3047, FHC3313, FHC3398, FHC3399, FHC3725, FHC3853, FHC3965, and FHC4027 (Neff *et al.* 1999; Breen *et al.* 2001; Guyon *et al.* 2003).

Genotypes were obtained by polymerase chain reaction (PCR) amplification using QIAGEN Multiplex PCR kits with a hybrid forward primer consisting of the published forward primer with an M13F (-20) sequence (16 bp) added to the 5' end and a fluorescent dye-labelled M13F (-20) primer (Boutin-Ganache *et al.* 2001). The reverse primer was unlabelled. Reactions were performed in 10 μ L volumes containing 1.5 μ L DNA, 1.0 μ L primer mix, 0.4 μ L 10 mg/mL bovine serum albumin, 5.0 μ L QIAGEN mastermix and double-distilled water. Loci were multiplexed in sets of two to five, using primer mix prepared according to the manufacturer's guidelines. Amplifications were performed on a Peltier Thermal Cycler (MJ Research PTC-200) using the multiplex cycling profile: 95 °C for 15 min; 25 cycles at 94 °C for 30 s, 59 °C for 90 s, and 72 °C for 60 s; then 20 cycles at 94 °C for 30 s, 53 °C for 90 s, and 72 °C for 60 s with a final extension at 60 °C for 30 min. PCR products were analysed on an ABI 3730XL capillary sequencer and alleles were analysed using ABI GENEMAPPER version 3.0 software (Applied Biosystems). Allele repeats were checked with Excel Microsatellite Toolkit (Parker 2001).

Genetic diversity

The total pedigree data set included 200 genotyped individuals with > 70% of the loci typed. We utilized population- and individual-based approaches for calculating heterozygosity. We used CERVUS (Marshall *et al.* 1998) for calculating population-based variation indices. The observed heterozygosity was obtained by dividing the total number

of heterozygotes by the total number of individuals typed and the multilocus expected heterozygosity was calculated and averaged across all loci using the unbiased formula of Nei (1987) from allele frequencies assuming Hardy-Weinberg equilibrium (Marshall *et al.* 1998). Uncorrected individual-based heterozygosity was the proportion of heterozygous loci typed for each individual and as in Bensch *et al.* (2006), was used to compare individuals, such as parent-offspring or breeding pairs and to investigate mate choice based on individual heterozygosities. Annual heterozygosities were calculated based on the calendar year for all living animals. We also estimated relatedness and inbreeding coefficients (F_{IS}) to assess trends in genetic diversity over the study period. We tested for significant deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) for individuals in the pedigree data set using the web version of GENEPOP version 3.4 (Raymond & Rousset 1995) with an adjusted P value corresponding to $\alpha = 0.05$ after Bonferroni correction (Rice 1989). We tested for the presence of null alleles using MICROCHECKER (Van Oosterhout *et al.* 2004). Inbreeding coefficients were calculated as population-based estimates with FSTAT version 2.9.3.2 (Goudet *et al.* 2002). Data were assessed for normality using quantile-quantile plots. Breeding pair relatedness was assessed with KINSHIP (Goodnight & Queller 1999).

To compare genetic diversity in the reintroduced population to that from alternative breeding strategies, we created breeding pools consisting of individuals with pedigreed ancestry for three scenarios: (i) open gene pool; (ii) restricted gene pool; and (iii) managed gene pool. The open gene pool scenario placed all reproductively mature individuals (> 2 years) in an annual breeding population without regard to kinship or pack membership. Under the restricted gene pool scenario, females were restricted to breeding with males from the same pack. This scenario presumed no inbreeding avoidance and reflected only a preference for mates in close proximity. Such a scenario may approximate conditions of low mate availability due to high pack isolation (such as after a long distance colonization event) or low pack density (Wayne *et al.* 1991; Schröder & Promberger 1993; Ellegren *et al.* 1996; Liberg *et al.* 2005). The managed gene pool was created following rules commonly used in captive breeding programmes: minimizing mean kinship, maximizing gene diversity, increasing population size and eliminating unknown pedigree lineages (Ballou *et al.* 2001; Frankham *et al.* 2002). Using PM2000 (Pollak *et al.* 2002) and MATERX (Ballou *et al.* 2001; Ralls & Ballou 2004), we identified ideal breeding pairs that would maximize these breeding goals according to the joint measure of the Mate suitability index (MSI). The MSI provides a value for each male-female pair based on how well the pairing maximizes genetic diversity while minimizing inbreeding, unknown ancestry and mean

kinship. Pairs were ranked on a scale of 1 (beneficial mate pair) to 6 (detrimental mate pair). Using default settings in *MATER_x*, we identified the same number of ideal mate pairs as were actually observed annually.

We used these breeding pools in a simulation to estimate heterozygosity of adults and offspring in 2004 with the simulation model *WOLFY* version 0.1 developed for this study and available online (<http://taylor0.biology.ucla.edu/~daearl/software/wolf/>). We focused on the year 2004 after the population reached carrying capacity and 24 male–female breeding pairs were observed. We chose 24 breeding pairs from each of the three breeding pools discussed above (restricted, open and managed) and allowed them to produce the average number of offspring observed in that year ($N = 6$) based on Mendelian inheritance of 26 loci (see Results). We then calculated the average individual heterozygosity of the parents and offspring (the new population). For the restricted scenario, each pack had at least one breeding pair. For each scenario, we simulated 24 breeding pairs for 1 million iterations by resampling males with replacement and females without replacement (assuming no multiple paternity of litters but allowing for males to fertilize multiple females). The resulting heterozygosity histogram is displayed as the frequency of individuals within each of 800 heterozygosity bins. The simulation was not intended to incorporate all possible complexities of wolf pack breeding structure; rather, this simulation was used to assess the effect on heterozygosity of random and restricted breeding scenarios without regard to kinship and managed breeding with regard to kinship across the entire breeding pool.

We determined the opportunities for inbreeding within a pack for the restricted breeding strategy data set. For each pack, we divided the number of possible male–female adult pairs ($= 2$ years old) between close relatives ($r > 0.25$) by the number of all possible adult pairings. We averaged these proportional values across packs for each year as a measure of possible breeding opportunities within packs that would constitute inbreeding.

Effective population size estimates and generation time estimate

We estimated annual effective population sizes (N_e) based only on those individuals genotyped and included in the pedigree data set ($N = 200$) with the population management software *PM2000* (Pollak *et al.* 2002). This method excludes individuals whose parentage assignments have not been resolved. We estimated the mean generation time using the population viability analysis (PVA) simulation program *VORTEX* (Lacy *et al.* 2005; Miller & Lacy 2005) and the observational life history and breeding demographic data for the 2004 population (Table S1, Supplementary material).

Parentage and pedigree reconstruction

We calculated the probability that two siblings would have identical genotypes by chance (PID_{sib}, as in Evett & Weir 1998; Waits *et al.* 2001) using the program *GIMLET* version 1.3.1 (Valière 2002). PID_{sib} is a conservative estimate of the power to resolve individuals given population substructure or when comparisons are made between related individuals (Waits *et al.* 2001). The population genealogy was determined by sibship and parentage analysis of 200 grey wolves. Sibship was inferred using *COLONY* version 1.3 to identify groups of full and half-sib offspring utilizing a maximum-likelihood approach for relationship estimation (Wang 2004). The groups that are produced include all individuals that share approximately 50% of their genes. From these groups, we identified putative full- and half-sib dyads as those sharing two and one parent, respectively. Dyads were then grouped to construct putative litters to reduce analytical complexity. This narrowed the pool of candidate parents for additional pups in parentage testing that had no parentage information. Parentage analyses and assignments for parent–offspring dyads were completed under a likelihood approach employed in *CERVUS* version 3.0 (Marshall *et al.* 1998). Parentage assignments were determined initially through exclusion with field and genetic data where possible followed by use of log likelihood (LOD) scores for candidate parents given the offspring genotypes and allele frequencies in the population. Simulations were performed to determine the likelihood of random individuals as parents and the ratio between this value and that for candidate individuals is expressed as the delta value. We considered delta values that were significant at 95% and 80% levels. To generate delta values, we simulated 10 000 offspring and 50 candidate males allowing for 20% of the population to be unsampled and 20% incomplete multilocus genotypes. *CERVUS* was also used to calculate the polymorphic information content (PIC) and the probability that a single-locus genotype is identical between two randomly chosen individuals.

We used two general approaches for resolving parentage: open and restricted. Open paternity/maternity analyses were utilized for individuals having unresolved sibship groups or had no a priori assessments of parentage based on field observations and involved testing for parentage using all reproductively mature males/females (> 2 years). Candidate parent pools were not based on geographical proximity to potential offspring as extra-pack copulations have been observed (Yellowstone Wolf Project, NPS, unpublished data). Restricted analyses used pools of candidate parents identified by sibship analysis or field observations to reduce the pool of potential parents and increase the certainty of parentage assignments that might be obscured by the presence of close relatives. Individuals were placed into pack pedigrees based on assigned parentage

and year of birth as determined by the age estimate of the individual at the time of sampling. The genealogies reported here include sampled individuals only and therefore do not completely reflect actual annual pack compositions and pup production. The genealogy was prepared with PEDIGREE VIEWER (Kinghorn 1994).

Breeding pair and interpack relatedness

Relatedness was assessed by likelihood simulations and significance testing with KINSHIP (Goodnight & Queller 1999). To corroborate inbreeding events identified by field and pedigree-based relationships, we evaluated relatedness among breeding pairs with likelihood simulations executed in KINSHIP, testing the hypothesis that breeding pairs are related at $r = 0.25$ or greater. We assessed interpack kinship ties for 2002 by use of the inferred genealogy at two relatedness levels: (i) $r = 0.5$, indicating first order relationships of parent–offspring or full-sibling; and (ii) $r = 0.25$, indicating second-order relationships such as half-sibling, aunt/uncle–nephew/niece, or grandparent–grand offspring. We choose 2002 because this year is well sampled and is representative of years when the population reached carrying capacity.

Long-term genetic trajectory predictions

The future genetic trajectory of the YNP wolves was investigated using the population viability analysis (PVA) simulation program VORTEX (Lacy *et al.* 2005; Miller & Lacy 2005). The demographic characteristics during 2004 and a carrying capacity of 170 individuals (see Results) were used as input parameters (Table S1). With respect to breeding system parameters, we found no significant difference between running short- and long-term monogamy in the model, and polygamy was not appropriate. We used long-term monogamy in the model because as a first order approximation, this adequately characterizes wolf-mating structure (Mech & Boitani 2003a). We assumed no immigration and evaluated 0, 5 and 10% emigration each year to represent observed one-way emigration of individuals from the park into the Greater Yellowstone Area. We simulated 100 years of population dynamics using 1000 iterations to estimate change in observed and expected genetic heterozygosity and in the inbreeding coefficient. We also investigated the population size required (assuming no immigration) to maintain heterozygosity at 95% of its current level over the next 100 years for a population with the observed demographic characteristics by increasing the VORTEX model population carrying capacity. Finally, we investigated the amount of immigration needed to prevent decreases in heterozygosity by adding wolves to the population each year using the supplementation scenario option in VORTEX, which assumes added individuals are unrelated to the current population.

Results

Observed demographic history of reintroduction

The Yellowstone population expanded rapidly after the reintroductions of 1995 and 1996. In total, 41 wild-born wolves were reintroduced from 1995 through 1997; 31 founders were from Canada and 10 from northwest Montana. The Montana individuals were derived from an independent Canadian stock and only three of these pups lived past 1 year with just two reproducing in the wild. Population growth was initially very high through the reintroduction phase (40–50% per year, 1995–1997) and continued to increase through 2003 (10–15% per year, 1999–2003) reaching a maximum of 174 wolves before declining to 169 wolves in 2004 (Smith 2005). Pack formation and territory establishment followed a similar pattern, increasing from three packs in 1995 and stabilizing at 16 packs in 2003 and 2004 (Fig. 1). Life table analysis calculated from age-specific birth and death rates of the Yellowstone population as of 2004 (Table S1) were used in VORTEX to estimate a mean generation time of 4.16 years (Miller & Lacy 2005). Since 2004, population size and the number of packs have stabilized, indicating that carrying capacity in Yellowstone has been reached (Yellowstone Wolf Project, NPS, unpublished data). Increasing interpack conflict and intra-specific mortality have been associated with higher wolf densities, suggesting that carrying capacity has been socially mediated (Yellowstone Wolf Project, NPS, unpublished data). Emigration of wolves from YNP was common throughout the study period, and was responsible for the establishment of packs in the GYA outside of the park (USFWS *et al.* 2005). Immigration of wolves to YNP from outside the recovery area was not observed (Yellowstone Wolf Project, NPS, unpublished data) or revealed from genetic studies of wolves within or outside the park (vonHoldt unpublished data; see below).

General trends in genetic diversity

Deviations from Hardy–Weinberg expectations occurred in less than 10% of the original 30 loci on average after a Bonferroni correction for multiple testing (see Table S2, Supplementary material). However, when inheritance patterns were examined with known field genealogies, four loci (Pez6, Pez11, 3313 and 4027) consistently exhibited non-Mendelian patterns of inheritance and were dropped from all subsequent analyses. The remaining 26 loci were polymorphic in each year, ranging from three to 18 alleles per locus with an allelic richness (A_R) of 6.3–9.1 alleles per locus for the pedigree population ($N = 200$; Table 1 and Table S3, Supplementary material). Tests were insignificant for the presence of null alleles for all 26 loci (using Bonferroni correction for multiple tests). Eighteen of 325

Table 1 Population census size (N_C), total number of Yellowstone packs (N_{Packs}), number of individual genotyped (N_g), observed/expected heterozygosity (H_O and H_E , respectively), allelic diversity (A_R), inbreeding coefficient (F_{IS}), pedigree-based effective population size estimates (N_e), and within-pack inbreeding opportunities for individuals in the reconstructed pedigree of Yellowstone National Park (1995–2004; $N = 200$; 26 microsatellite loci)

	1995	1996†	1997	1998	1999	2000	2001	2002	2003	2004
Population N_C *	21	37	80	83	72	119	132	148	174	169
N_{Packs}	3	8	8	8	7	9	10	14	14	16
N_g †	21	45	69	62	52	67	65	65	61	66
Mean H_O	0.694	0.698	0.702	0.697	0.698	0.714	0.723	0.727	0.721	0.725
Mean H_E	0.717	0.754	0.760	0.750	0.740	0.744	0.740	0.735	0.733	0.737
A_R	6.3	8.8	9.1	9.0	8.6	9.0	8.7	8.7	8.5	8.6
F_{IS}	-0.063	-0.016	-0.012	-0.026	-0.044	-0.043	-0.052	-0.054	-0.050	-0.051
N_e	6	13.7	22.6	16.9	16.3	17.3	16.3	14.4	17.1	22.1
N_e/N_g	0.29	0.30	0.33	0.27	0.31	0.26	0.25	0.22	0.28	0.33
No. of total within pack possible male–female pairs	8	14	20	20	37	38	39	60	56	100
Within-pack inbreeding opportunities	0.00	0.14	0.10	0.10	0.24	0.24	0.13	0.35	0.55	0.59

*Annual census size as of 31 December.

†Individuals in the pedigree data set having at least 70% of the 26 loci genotyped.

‡Includes individuals that died before annual census count.

pairwise comparisons showed significant linkage disequilibrium (LD) following Bonferroni correction ($P < 0.05$) but none of the 18 pairings were loci located on the same chromosome, suggesting that LD is probably due to population structure rather than physical linkage. Thus, we included all remaining 26 loci in our analyses.

With an increase in allelic diversity when the second founder population was introduced in 1996, genetic diversity remained high and inbreeding was near zero for the entire study period. Observed heterozygosity was maintained in the pedigree population at high levels ($H_{1995} = 0.694$; $H_{2004} = 0.725$) whereas inbreeding (F_{IS}) has only increased slightly over time (1995 $F_{IS} = -0.063$; 2004 $F_{IS} = -0.051$), results that are consistent with the rapid population expansion ($N_{1995} = 21$; $N_{2004} = 169$; Table 1 and Fig. 1). The marked increase in the inbreeding coefficient observed in 1996 and 1997 was associated with the addition of a litter from a full-sib inbreeding event of wolves housed in the same pen (1996 $F_{IS} = -0.016$; 1997 $F_{IS} = -0.012$, see below).

Mean levels of heterozygosity for the observed breeding pool over the 10-year study were not significantly different from those chosen in the managed breeding scenario (see Methods; $H_{\text{Observed}} = 0.750$, $SE = 0.013$; $H_{\text{Managed}} = 0.761$, $SE = 0.017$; Fig. 2A). Relatedness of the observed breeders is higher for the first 4 years (1995–1998) than the managed scenario, and significantly higher for two of these years (Fig. 2B). Thereafter, values are similar except for the last 2 years when relatedness is significantly higher for the observed population (2003; $r_{\text{Observed}} = 0.017$, $SE = 0.002$; $r_{\text{Managed}} = -0.009$, $SE = 0.007$; 2004; $r_{\text{Observed}} = 0.010$,

$SE = 0.002$; $r_{\text{Managed}} = -0.009$, $SE = 0.006$; Fig. 2B). Mean inbreeding coefficients (F_{IS}) of breeders decreased over time for both scenarios but were more often lower in the managed population ($F_{\text{Observed}} = 0.012$, $SE = 0.023$; $F_{\text{Managed}} = -0.011$, $SE = 0.022$; Fig. 2C).

Finally, we simulated breeding in 2004 to compare heterozygosities of open, restricted and managed breeding scenarios (Fig. 3). The mean heterozygosity of the observed population ($H_{\text{Observed}} = 0.750$, $SD = 0.013$) was higher than the restricted ($H_{\text{Restricted}} = 0.690$, $SD = 0.019$) and open breeding strategies ($H_{\text{Open}} = 0.728$, $SD = 0.026$) and not significantly different from the managed scenario ($H_{\text{Managed}} = 0.764$, $SD = 0.008$). High heterozygosity in the observed population can be attributed to the active choice of wolves to breed with unrelated individuals within or outside of their natal pack (see below).

Sibship groups and parentage assignments

From sibship analysis, we identified 52 sibship groups consisting of multiple individuals from 14 packs. From these groupings we resolved 31 full-sib dyad relationships and 19 half-sib dyads based on sharing of one parent, with only two dyads being unresolved. All genetically deduced sib groupings were consistent with field data. The 26 microsatellites in our data set had a very low overall probability of identity among siblings (PID_{sib} ranged from 9.33×10^{-13} in 1996 to 2.91×10^{-12} in 2004). This result implies that full-siblings sharing the same genotype by chance were highly unlikely in our population.

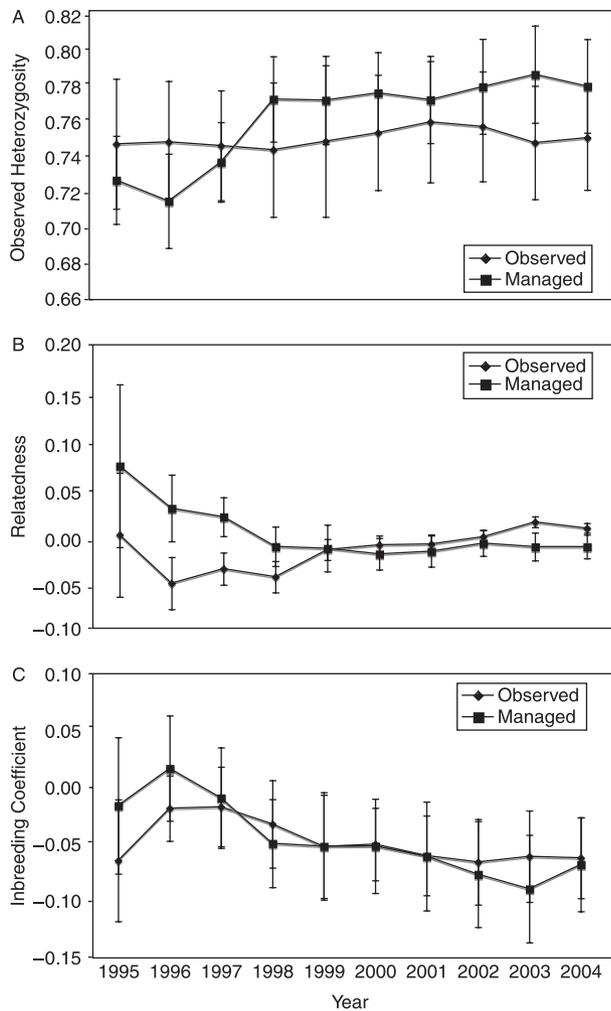


Fig. 2 Annual heterozygosity (A), relatedness (B) and inbreeding coefficient (C) for the observed breeding population as compared to breeders selected by $MATER_x$ under a managed breeding strategy. Error bars represent 1.96 standard deviations from the mean and significance is defined by mean values being separated by more than two standard errors.

Initial a priori field-based (nongenetic) parentage data resolved 12 two-parent, eight paternity and 22 maternity assignments. Genetic analyses resolved parentage for 200 individuals, 183 assignments (91.5%) at the 95% confidence level and 17 (8.5%) at the 80% confidence level. We found no multiple-paternity within a litter. The polymorphic information content was high ($PI_C = 0.733$) and probabilities of nonexclusion were on the order of 10^{-12} for the set of 26 loci. Of the 200 individuals, genetic parentage analyses resolved 126 (77.3%) offspring with two-parent assignments and 37 (22.7%) offspring having single-parent assignments (28 paternity and nine maternity assignments). Five two-parent assignments were resolved by sibship reconstruction. There were unresolved parentage

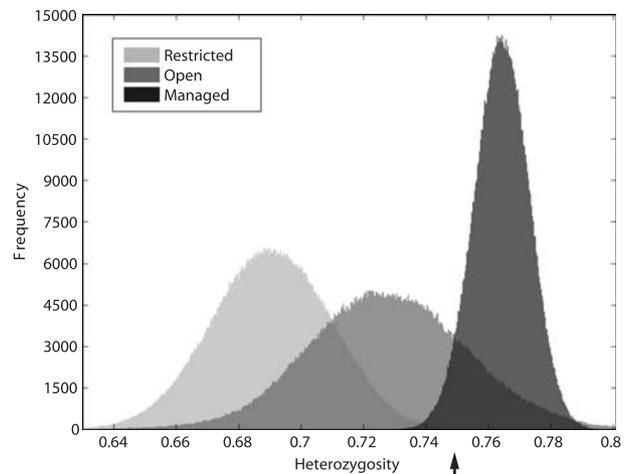


Fig. 3 Histograms of the average heterozygosity for simulated populations of parent and offspring (for 1 million iterations) for open, restricted, and managed breeding scenarios compared with observed breeding strategy (arrow) in 2004.

assignments for seven paternities with known maternity (two due to unsampled males) and 15 maternities with known paternity (seven due to unsampled females). The 10 Sawtooth pups were assumed to be full-sibs and were included in all parentage analyses.

Relatedness of breeding pairs

We determined the relatedness of 31 breeding pairs using field and genetic confirmation of parentage, and found 28 (90%) were unrelated. However, only two of the three related mating events represent a natural event (see below); hence, the rate of naturally occurring unrelated matings is 93%. The mean (\pm SE) pairwise relatedness values between breeding pairs was -0.026 ± 0.03 (range -0.313 – 0.515 , $N = 31$) and their mean (\pm SE) inbreeding coefficient was $F = -0.005 \pm 0.007$ (range -0.087 – 0.084 , $N = 31$; Table 2). Breeding pairs had high mean levels of heterozygosity ($H_{\text{parents}} = 0.787 \pm 0.015$) that differed from their offspring ($H_{\text{pups}} = 0.741 \pm 0.014$, $N = 151$; pairwise t -test: $t = 2.12$, d.f. = 30, $P = 0.043$; Table 2). Further, we partitioned the data set into known breeding ($N = 65$) and nonbreeding ($N = 135$) individuals and found no significant differences between groups in heterozygosity ($H_{\text{breeding}} = 0.749$, SE = 0.010; $H_{\text{nonbreeding}} = 0.727$, SE = 0.010, $t = 1.543$, d.f. = 164, $P = 0.125$). Consequently, this latter result does not support a bias toward matings of individuals with higher heterozygosity as found in inbred wolf populations (Bensch *et al.* 2006).

The mechanism of formation for 34 breeding pairs was documented (Table 3). Five breeding pairs were established prior to their release, with 29 other pairs forming in

Table 2 Parental and offspring observed heterozygosity (H_O), number of genetically verified offspring ($N_{\text{offspring}}$) and the pedigree-based inbreeding coefficient (F) of the breeding pair ($N = 31$)

Sire	Dam	Pack	Mean parental H_O	Relatedness	$N_{\text{offspring}}$	Mean offspring H_O	$F_{\text{BreedPair}}$
2M	7F	Leopold	0.720	-0.167	25	0.727	0.010
4M	5F	Mollie's	0.654	-0.033	3	0.780	-0.028
6M	5F	Mollie's	0.654	0.227*	4	0.660	0.022
8M	19F	Rose Creek	0.965	-0.145	1	0.769	0.019
10M	9F	Rose Creek	0.850	-0.065	6	0.766	-0.027
13M	14F	Yellowstone Delta	0.876	-0.110	6	0.798	-0.044
21M	286F	Druid Peak	0.825	0.172†	2	0.563	0.044
21M	40F	Druid Peak	0.825	-0.213	4	0.682	-0.014
21M	42F	Druid Peak	0.820	-0.037	7	0.720	-0.010
2M	106F	Druid Peak	0.902	-0.188	6	0.776	0.043
28M	27F	Nez Perce	0.750	-0.135	6	0.813	-0.057
29M	37F	Nez Perce	0.704	0.515‡	1	0.550	0.084
34M	16F	Chief Joseph	0.908	0.008	2	0.635	-0.016
34M	33F	Chief Joseph	0.734	-0.154	6	0.852	-0.087
34M	17F	Chief Joseph	0.784	-0.046	2	0.708	-0.005
35M	30F	Thorofare	0.844	-0.186	2	0.827	-0.043
38M	41F	Druid Peak	0.692	0.073	3	0.694	-0.049
38M	42F	Druid Peak	0.706	0.039	1	0.885	-0.063
70M	48F	Nez Perce	0.673	-0.313	4	0.828	-0.033
72M	48F	Nez Perce	0.685	0.082	11	0.784	-0.005
120M	14F	Yellowstone Delta	0.759	-0.049	4	0.672	-0.020
165M	16F	Sheep Mountain	0.965	-0.008	4	0.691	0.015
205M	152F	Swan Lake	0.778	-0.034	1	0.750	-0.005
206M	152F	Swan Lake	0.686	0.130	4	0.748	-0.025
227M	106F	Geode Creek	0.782	-0.005	2	0.839	0.029
294M	106F	Geode Creek	0.817	-0.097	2	0.763	0.015
301M	251F	Agate Creek	0.813	-0.005	1	0.808	0.004
302M	255F	Druid Peak	0.824	-0.039	1	0.708	0.035
303M	151F	Cougar Creek	0.761	0.093	7	0.680	0.029
487M	126F	Yellowstone Delta	0.812	-0.099	2	0.776	0.000
534M	209F	Leopold	0.827	-0.005	1	0.731	0.039
Average			0.787	-0.026	4.2	0.741	-0.005

*Unknown ancestry and probably an aunt–nephew mating ($r = 0.25$; $P < 0.05$).

†Unknown ancestry ($r = 0.25$; $P < 0.05$).

‡Full-sib mating in acclimation pen prior to release ($r = 0.25$; $P < 0.001$).

the wild in YNP. Two of these naturally forming pairs (7%) were lone individuals that joined in 1996 (2M and 7M of Leopold, Fig 4; 35M and 30F of Thorofare pack), whereas the remaining 27 pairs (93%) formed in the context of a group. On six occasions, the vacancy created by the death of a dominant male breeder was filled by migration into the pack of an unrelated male, while no such events were documented for females. These events explained six pairs that formed as the result of the dominant female breeding the new male, as well as five subordinate females breeding with the new male. In total, we documented nine cases of subordinate individuals breeding, all of which were females related to the dominant female breeder as siblings, daughters or nieces. The Druid Peak pack exemplified a highly complex, multiple-breeding pack structure in which heter-

ozygosity was maintained by these mechanisms (Fig. 4). For example, a male immigrant (21M) filled a vacant breeding position in 1997 after the death of the dominant male (38M), subsequently breeding unrelated females for multiple years until the pack split in 2001. There was an increase in heterozygosity through time associated with these years of multiple breeding ($H_{1997} = 0.743$, $H_{2001} = 0.778$; Fig. 4).

We genetically confirmed three extra-pack copulations when subordinate females formed temporary liaisons with interloping males during the breeding season, all in the Druid Peak pack. The first case was in 2002 when we confirmed parentage of at least one offspring by a dispersing Nez Perce male (214M) who paired temporarily with an unmarked subordinate female but did not join the

Table 3 Observed mechanisms for first-time breeding pair formation in Yellowstone National Park for 32 pairings (1995–2004)*

Category	Male	Female	Total
Reintroduced pair			4
Two dispersing individuals join			2
Multiple individuals join and at least two breed			5
Within-pack inheritance/succession of dominant breeder position	2	2	4
Immigrant usurps an active breeder	1		1
Immigrant assumes vacant dominant breeder position	6		6
Dominant breeds new immigrant		6	6
Dominant breeds subordinate	7		7
Subordinate breeder in natal pack		9	9
Interloper breeds subordinate but does not join pack	3		3

*Includes only genotyped pairs where category was certain (not including inbreeding events).

pack (Fig. 4). A female offspring (286F) from this pairing then bred in the Druid Peak pack in 2004 with the alpha male, resulting in one of the two naturally occurring inbreeding events (see below; Table 2; Fig. 4). In 2003, we confirmed paternity by a dispersing male (302M) from the Leopold pack who fathered at least three pups with two different subordinate females in the Druid Peak pack before joining the pack in 2004 (Fig. 4).

Five breeding pairs formed in the context of a group outside of an established pack and involved an individual disperser joined by an opposite-sex group of wolves. This was the primary mechanism for forming new packs in Yellowstone after 1996 (see below). Four pairs formed as an individual inherited the dominant breeding position in their natal pack, and in each of these cases, the dominant, opposite-sex breeder was not their relative. We documented one event of a male disperser usurping the breeding position from a long-term dominant male. This immigrant male (534M) from the Nez Perce pack forced the long-time dominant male breeder (2M) to leave the pack (Fig. 5). The new male's subsequent breeding was associated with an increase in pack heterozygosity ($H_{2001} = 0.729$; $H_{2004} = 0.743$). These results demonstrate remarkable flexibility in the means by which pairs form and reveal a greater diversity of mechanisms within a single population than previously documented in other studies (e.g. Rothman & Mech 1979; Hayes *et al.* 1991; Mech & Boitani 2003a). However, despite this variability, all naturally observed mechanisms avoided breeding between highly related individuals (see below) and were often associated with increased heterozygosity in packs.

Inbreeding

Over the 10-year study, we documented only three breeding pairs that were significantly related ($r = 0.515$, $P < 0.001$; $r = 0.227$, $P < 0.05$; $r = 0.172$, $P < 0.05$). One was a probable aunt–nephew mating in the Crystal Creek pack, a second was between a probable grandfather–granddaughter mating in the Druid Peak pack, and the third was a full-sib mating in Nez Perce pack (Table 2). The probable aunt–nephew inbred pair was significantly related but the exact relationships were not resolved due to unknown ancestry. However, this pairing occurred under extenuating circumstances as the female's unrelated mate died prior to the breeding season, leaving her nephew as the only remaining wolf in the pack during the breeding season in 1997, a year of low mate availability. The probable grandfather–granddaughter pair was significantly related, and field observations indicate that the shared relative was the breeding female's unsampled mother who was presumably a daughter of the breeding male (Table 2; Fig. 4). This pairing occurred immediately following the death of the breeding male's former long-term mate at the peak of the breeding season, leaving only related females as possible mates. The third mating reflected human interference as two full-sibs from the Nez Perce pack were penned in 1997 to act as surrogate parents for 10 orphaned pups from the Sawtooth pack of northwest Montana (Fig. 6). This resulted in the only full-sib breeding event in Yellowstone National Park in the 10-year period. The heterozygosity of the Nez Perce pack was initially high on reintroduction ($H_{1996} = 0.802$), then reduced as a consequence of this full-sib mating event the next year ($H_{1997} = 0.753$), but remained stable with the inclusion of the unrelated Sawtooth wolves ($H_{1997} = 0.790$; Fig. 6). Only two of these Sawtooth individuals (70M and 72M) acquired breeding status in the wild, both with the tenured Nez Perce dominant female (48F) that maintained heterozygosity over subsequent years ($H_{2000} = 0.786$ to $H_{2004} = 0.813$).

In 1997, the proportion of possible breeding opportunities within packs that would constitute inbreeding was 10%, increasing over subsequent years as the population expanded and peaking in 2004 at 59% as relatives accumulated within packs (Table 1). Consequently, Yellowstone wolves actively avoided breeding with close relatives, as no naturally occurring inbreeding events were documented between individuals with $r > 0.25$ despite an increasing opportunity to do so.

New pack formation

The formation of new packs always involved the establishment of breeding pairs, as they are the fundamental unit of wolf social structure (Murie 1944; Mech 1970). Seven packs were established during the reintroduction (Chief

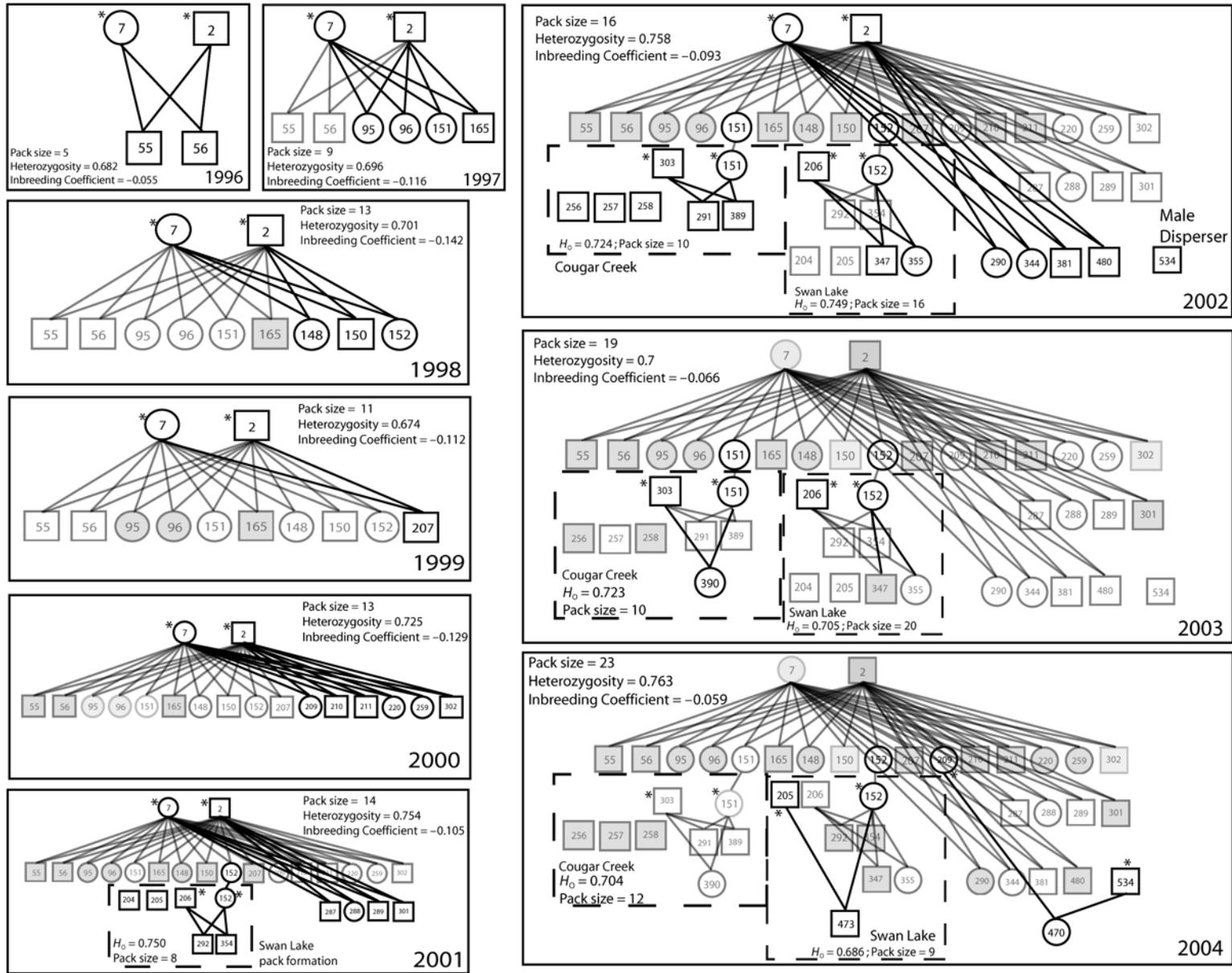


Fig. 5 Leopold pack genealogy of genotyped individuals. Circles represent females and squares represent males. Pack size reflects end-of-year count. Asterisk indicates dominant individual; shaded symbols represent death, dispersal or presence unknown. (H_o , observed heterozygosity).

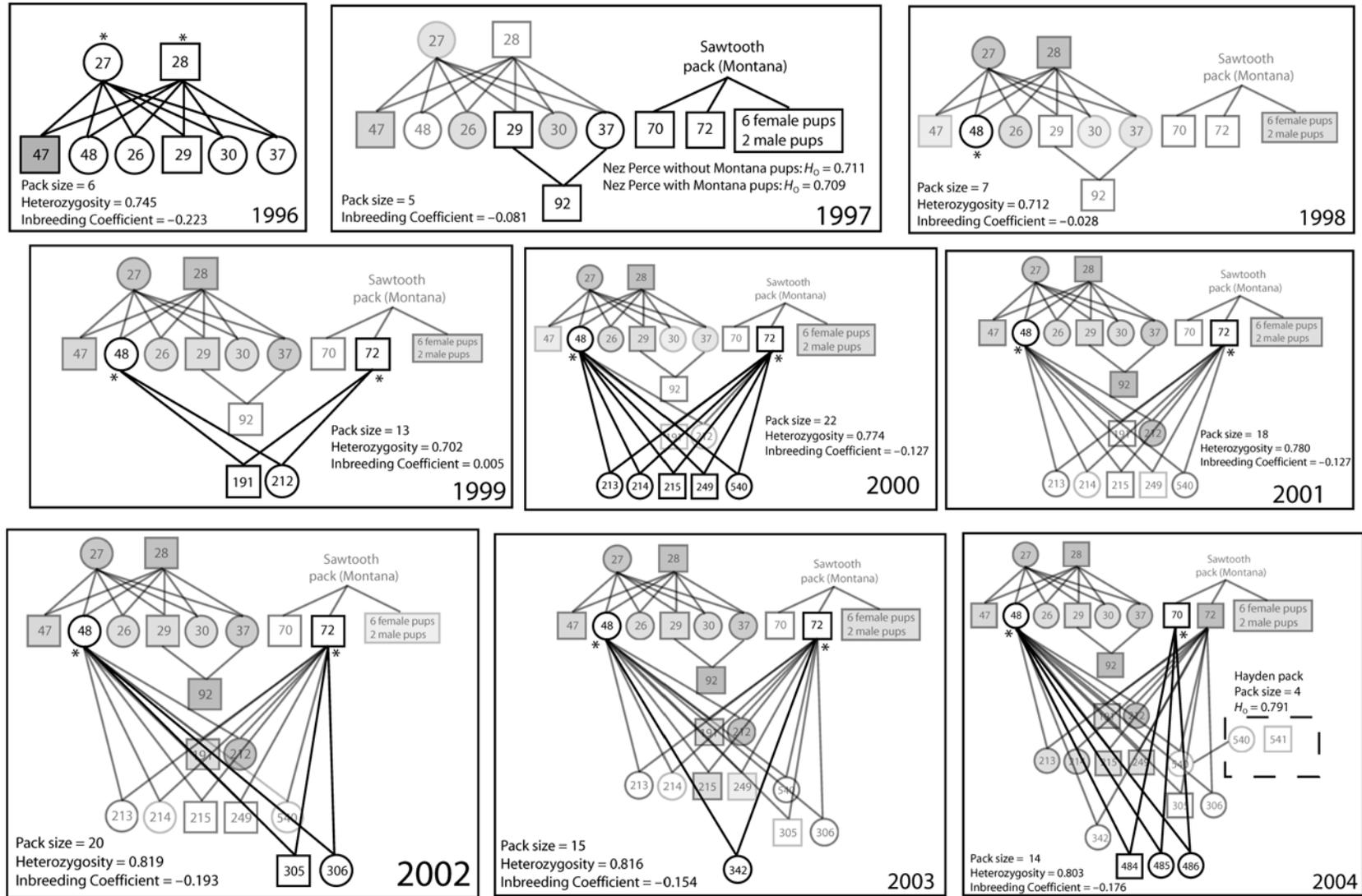


Fig. 6 Nez Perce pack genealogy of genotyped individuals. Circles represent females and squares represent males. Pack size reflects end-of-year count. Asterisk indicates dominant individual; shaded symbols represent death, dispersal or presence unknown. (H_o , observed heterozygosity).

Joseph, Crystal Creek, Druid Peak, Lone Star, Nez Perce, Rose Creek, and Soda Butte) with four of them maintaining core group membership throughout all 10 years of the study (Fig. 1). Of the 15 naturally formed packs in subsequent years, only four (27%) of them resulted from the pairing of singletons (Bechler, Hayden, Leopold, and Thorofare), with two of these pairs genetically confirmed as being unrelated (Leopold and Thorofare; Fig. 1). Eleven (73%) new packs formed due to pack splitting (Agate Creek, Biscuit Basin, Buffalo Fork, Cougar Creek, Geode Creek, Gibbon Meadows, Sheep Mountain, Slough Creek, Specimen Creek, Swan Lake, and Tower). Pack splitting is defined here as a group of wolves leaving the pack together and joining with other dispersing individuals who then establish a new territory (Mech & Boitani 2003a). In five of these packs formed by splitting (Agate Creek, Cougar Creek, Geode Creek, Sheep Mountain, and Swan Lake), we genetically confirmed same-sex siblings or parent-offspring groups joining with unrelated individuals. Despite the fact that close relatives were involved in the formation of new packs, the breeders always mated with unrelated individuals preventing inbreeding.

The history of the Druid Peak pack exemplifies these trends. This pack rapidly expanded between 1997 and 2001, largely due to several years of multiple litters and high pup survivorship (Fig. 4). As a result, four new packs (Agate Creek, Buffalo Fork, Geode Creek, and Slough Creek) formed as groups of Druid wolves (comprised largely of female relatives) left and joined with unrelated individual males or groups of male relatives. Documented heterozygosities for three of these packs were high upon formation, as breeding pairs were comprised of unrelated individuals ($H_{\text{Agate}} = 0.734$, $H_{\text{Slough}} = 0.673$, $H_{\text{Geode}} = 0.787$). In contrast to Druid Peak pack's complexity, the Leopold pack conformed to the traditional structure of a monogamous breeding pair and their offspring (Fig. 5). Solitary female dispersers from Leopold, however, led to two new pack formations (Swan Lake and Cougar Creek) as groups of non-Leopold brothers splitting from their natal packs joined the females. These newly formed packs had high genetic variation ($H_{\text{Swan}} = 0.757$, $H_{\text{Cougar}} = 0.691$) and maintained relatively stable levels of heterozygosity throughout their tenure associated with the breeding of unrelated individuals as documented in 2004 ($H_{\text{Swan}} = 0.759$, $H_{\text{Cougar}} = 0.667$). These results are in contrast to past observations that most wolf packs form by two unrelated individuals joining (e.g. Rothman & Mech 1979; Mech & Boitani 2003a).

Interpack relatedness and breeder dispersal

Based on the genealogical relationships in 2002, we identified 162 (55%) kinships ties of 296 possible interpack relatedness comparisons ($r = 0.5$ and $r = 0.25$). We documented a total of 90 (56%) kinship ties having $r = 0.5$ between individuals

of different packs and 72 (44%) kinship ties with $r = 0.25$ in YNP in 2002 (Fig. 7). In that year, we found no ties between any of the packs with the Yellowstone Delta pack. The majority (94%) of the interpack kinship ties were between adjacent packs and only four ties with $r = 0.5$ and five ties with $r = 0.25$ joined two individuals of nonadjacent packs. For example, the Bechler pack was founded by a male that was a sibling and offspring of individuals born in the geographically nonproximate Rose Creek pack (Fig. 7). Kinship ties that spanned beyond adjacent territories were primarily the result of dispersing males becoming breeders in new or already established packs. In contrast, kinship ties between adjacent packs largely reflected female dispersal or female kin groups splitting from natal packs and establishing adjacent territories. Of the 90 kinship ties with $r = 0.5$, 18 (20%) were parent-offspring relationships and 72 ties (80%) were full-sibling relationships, both reflecting prior dispersal events from natal packs. One $r = 0.5$ kinship tie (1%) was due to an extra-pack copulation.

Overall, we documented a strong sex bias of dispersal into a pack as a breeder, with all successful immigrant breeders being males. For example, male 21M dispersed into Druid Peak pack in 1997 and male 227M dispersed into Geode Creek pack in 2004 (Fig. 4). In contrast, we documented no females dispersing into an already established pack and breeding. Alternatively, females became subordinate breeders in their natal pack or dominant breeders through new pack formation. Females did, however, disperse as singletons and joined with groups of males. For example, in 2000, dispersing Leopold female 152F joined with at least three male siblings (204M, 205M, 206M) from Chief Joseph pack to form the Swan Lake pack. The following year, Leopold disperser 151F joined with at least three male siblings (256M, 257M, 258M) also from the Chief Joseph pack, forming the Cougar Creek pack. Finally, we found no evidence for gene flow into YNP from outside as all individuals in the pedigree had ancestry derived from the population founders.

Effective population size estimates

We calculated effective population size (N_e) from the pedigree data set (Table 1). N_e increased with increasing population size after the founding events (1995 $N_e = 6$; 1996 $N_e = 13.7$; 2000 $N_e = 17.3$; 2004 $N_e = 22.1$). Using the ratio of N_e to the genotyped population size (N_g ; see Table 1), however, N_e/N_g ratio estimates did not change appreciably after the founding events (1996 $N_e/N_g = 0.30$; 1997 $N_e/N_g = 0.33$; 2000 $N_e/N_g = 0.26$; 2004 $N_e/N_g = 0.33$).

Long-term genetic trajectory prediction

As expected for an isolated small population of constant size ($N = 170$), our simulations predict a decrease in genetic

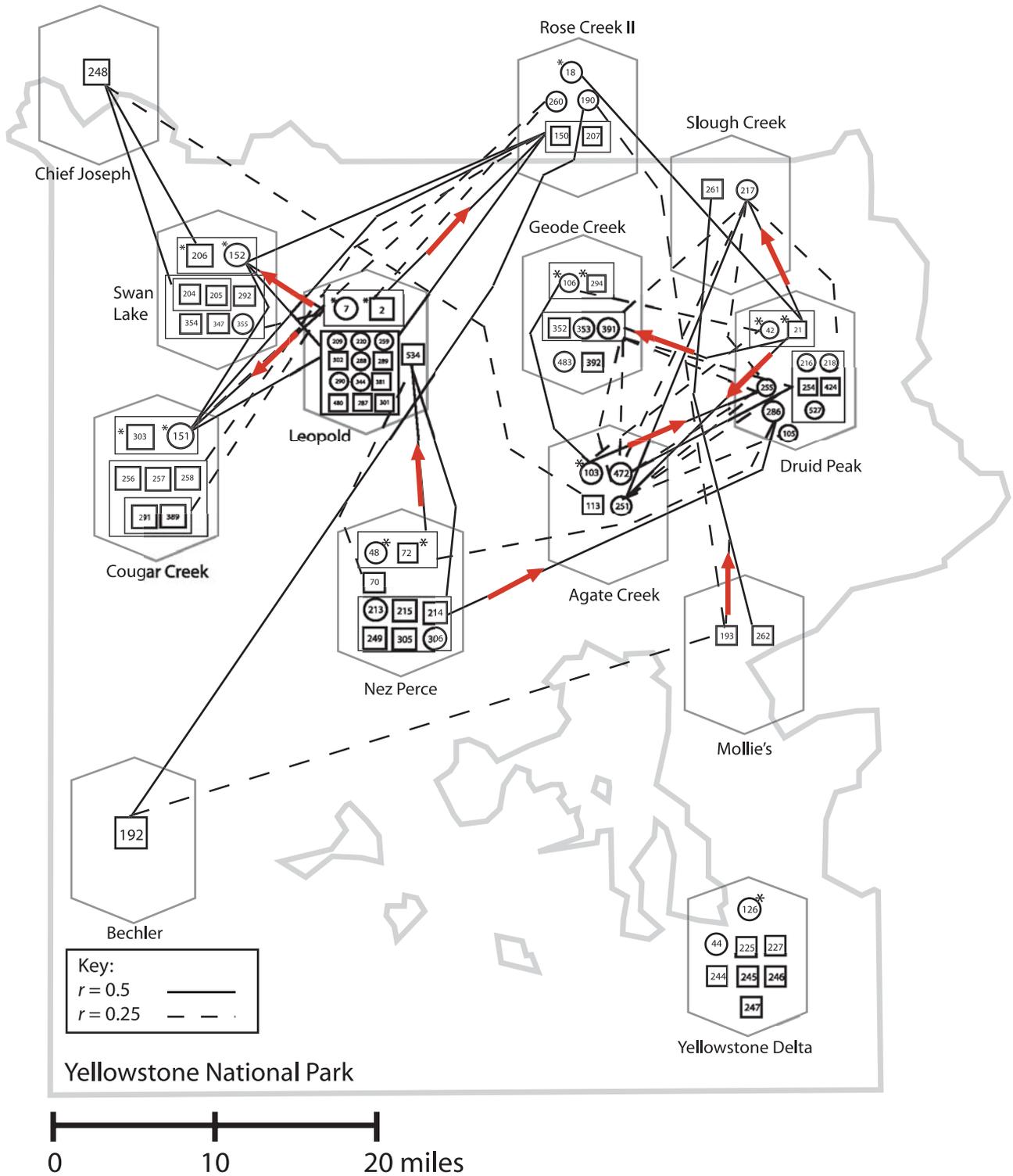


Fig. 7 Interpack relatedness for 2002 based on pedigree data. Arrows represent parent-offspring relationships, and point towards the offspring. Boxes contain either full-siblings or dominant pairs (asterisk) for interpack comparisons. This figure does not represent the census population, as only individuals and packs with known lineages are shown.

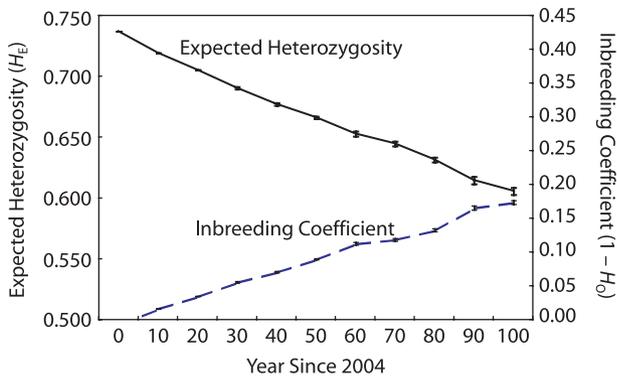


Fig. 8 Predicted changes in heterozygosity (H_E) and inbreeding coefficient (F_{IS}) of Yellowstone wolves assuming a constant population size ($N = 170$) and no gene flow using VORTEX (1000 iterations). Input parameters were based on Yellowstone's 2004 demographic and pedigree data. Error bars denote standard error.

heterozygosity and increase in inbreeding coefficient over the next 100 years (Fig. 8). Compared to the population genetic heterozygosity in 2004 ($H_E = 0.74$), the VORTEX simulation analysis predicts a decrease of 18.1% over 100 years to 0.60. The estimated inbreeding coefficient increases from -0.007 – 0.174 . For a constant-size isolated population of similar demography and life history, it is predicted that a population size of approximately 600 individuals would be needed to prevent a decrease in heterozygosity and increase in the inbreeding coefficients by less than 5% over 100 years (Table S3). Simulating the observed one-way migration of individuals out of YNP into the GYA indicates that the effect of 10% emigration per year decreases heterozygosity and increases the inbreeding coefficient by less than 0.5%, thus not having a significant impact on genetic variability. Simulation results also predict that immigration on the order of 12 individuals per year would be required to prevent significant decreases (<1%) in heterozygosity and increase in inbreeding coefficient (Table S4, Supplementary material). Further, based on observed N_e/N_c ratios of approximately 0.3 for the constant YNP population, these results predict that approximately four immigrating individuals per year would need to become breeders to maintain the genetic diversity of the Yellowstone population.

Discussion

Temporal genetic dynamics

Genetic diversity is reduced in small, isolated populations through increased drift and inbreeding and lack of migration from elsewhere (Taylor *et al.* 1994; Eldridge *et al.* 1999). Previous empirical studies of an isolated Swedish grey wolf population found that heterozygosity was lost at

a rate of 2% per generation (Bensch *et al.* 2006). In Yellowstone, theory predicts a loss of heterozygosity of about $1/2N_e$ per generation and a corresponding increase in the inbreeding coefficient (Hartl & Clark 1997). Consequently, given a harmonic mean of effective population size close to 52 and generation time of 4 years (Table 1), we would expect heterozygosity to have decreased by about 2.4% and inbreeding coefficients to have increased by about the same fraction. However, despite an absence of gene flow into Yellowstone, we found no temporal decrease in genetic variability or increase in inbreeding coefficients over 10 years or 2.5 generations (Table 1). The observed heterozygosity in Yellowstone is similar and in some cases higher than previous estimates for grey wolf populations (e.g. Forbes & Boyd 1996, 1997; Jedrzejewski *et al.* 2005). The inbreeding coefficients are far lower than the values of 0.41 found in the inbred wolf population of Scandinavia (Liberg *et al.* 2005; Bensch *et al.* 2006) or as Hedrick *et al.* (1997) found in three Mexican wolf lineages ranging from 0.184 to 0.608. We suggest the maintenance of genetic variation in Yellowstone reflects the large founding size and rapid population expansion (Sugg *et al.* 1996; Toro *et al.* 2003; Alvarez *et al.* 2005) as well as specific mechanisms to avoid inbreeding with close relatives. Inbreeding avoidance may enhance genetic variability beyond that predicted by simple genetic models (Hartl & Clark 1997; Keller & Waller 2002; Saccheri & Brakefield 2002; Vilà *et al.* 2003; Hogg *et al.* 2006).

Population assembly rules

We identified several factors governing the preservation of genetic variation that are important to a successful reintroduction. First, the founding population must be large and genetically diverse, a method preferred over repeated translocations over time (Wolf *et al.* 1996, 1998; Miller *et al.* 1999). A founding population in Yellowstone was established by 31 individuals from different packs belonging to two source populations in Canada. Additionally, 10 genetically distinct individuals were translocated from a northwestern Montana population early in the recovery process providing new genetic variation. The role that founding population size played in preserving high diversity in YNP is unique, as adequate number of founders are not common for reestablished wolf populations (Wayne *et al.* 1991; Hedrick *et al.* 1997; Liberg *et al.* 2005). Our results confirm the insight of the decision to select a large and diverse founding population for reintroduction (Forbes & Boyd 1997). Therefore, recovery programmes need to emphasize plans for an adequate number of founders to allow for the preservation of genetic diversity.

Second, there is clear evidence that given the choice, wolves avoid breeding with close relatives within their

natal pack or elsewhere. We found that of 30 natural matings, none involved pairings between pack members at the sibship or parent-offspring level ($r = 0.5$) and only two pairs were more distantly related ($r > 0.25$). Avoidance of close incestuous matings occurred despite increasing opportunities that such matings would occur without active behavioural avoidance of inbreeding. Evidence from previous genetic studies on natural wolf populations showed that when given a choice, pairs consist of unrelated individuals (Smith *et al.* 1997), thereby preventing loss of heterozygosity through inbreeding (Wright 1922; 1931; Chesser 1991a). However, our study is unique for showing that inbreeding avoidance occurs across a wide variety of mating strategies and contributed to the maintenance high levels of variation. Thus, as was part of this original reintroduction design, future reintroductions and population management should focus on providing opportunities for wolves to avoid inbreeding through actions such as reintroduction of a genetically diverse founding stock to areas of high quality habitat where several wolf packs can coexist in adjacent territories. Promoting the formation of several packs by introduction of unrelated mated pairs might also assist in providing future generations with opportunities to avoid inbreeding. Further, efforts to facilitate immigration from other populations will increase the pool of unrelated individuals who can occupy breeding positions or territories.

Third, breeding pairs can form under a wide variety of different mechanisms when sufficient opportunities are available. Previous studies have shown that the primary method of breeding pair formation in grey wolves involves single wolves meeting and breeding in both recolonizing and established populations (Rothman & Mech 1979; Fritts & Mech 1981; Peterson *et al.* 1984; Hayes *et al.* 1991; Bergerud & Elliott 1998; Hayes & Harestad 2000). We find that only 7% of confirmed pairings have formed in this way (Table 3). The mechanisms that describe how Yellowstone wolves obtained mates included: (i) utilizing a breeding vacancy within a natal or neighbouring pack; (ii) becoming a subordinate breeder; (iii) joining with a group of wolves from either their natal or different pack; and (iv) usurping an established breeder. All of these methods have been previously documented in other wolf systems (Mech & Boitani 2003a), but not to the extent we observe in YNP. This diversity may be an artefact of the reintroduction and rapid population expansion in a prey-abundant ecosystem devoid of wolves, or reflect the unprecedented detail to which wolves were monitored in YNP. Regardless, we show that diversity in pair formation mechanisms in Yellowstone contributes to the maintenance of high heterozygosity. For example, we found that vacant male breeding positions were filled primarily by unrelated immigrants, resulting in subordinate female breeders producing litters unrelated to the previous dominant male

breeder. The generality of this result is supported by a study of wolves from the Białowieża Primeval Forest where successors of breeding males were typically immigrant males in contrast to females who commonly obtained breeding positions within their natal pack (Jedrzejewski *et al.* 2005). Our study confirmed parentage and inbreeding avoidance in one of the most extreme cases of multiple breeding documented in a wild wolf population (Fig. 4), where the immigration of an unrelated male (21M) to the Druid Peak pack in 1997 led to breeding with multiple females in the pack for a series of years (Stahler *et al.* 2002).

This diversity of mating mechanisms may reflect conditions related to interpack competition or ecological constraints associated with dispersal (Brown 1974; Stacey 1979; Emlen 1982; Goldizen *et al.* 2002). For example, becoming a subordinate breeder or inheriting a dominant breeding position, in addition to the benefits received through group hunting and having a territory, may reflect the benefits of philopatry in a saturated landscape. Additionally, intraspecific strife has been the main cause of natural mortality for Yellowstone wolves (Smith 2005), and singletons are presumably at much greater risk during intraspecific interactions than individuals in a group (Yellowstone Wolf Project, NPS, unpublished data). Pack splitting may therefore reflect a less risky strategy for establishing territories as a larger group is more likely than singletons to establish a territory in a saturated landscape (Yellowstone Wolf Project, NPS, unpublished data). As with many species, variation in mating behaviour is presumably facilitated through mechanisms of asymmetric mate choice, dispersal and extra-group/pair copulations (Pusey & Wolf 1996; Smith *et al.* 1997; Ross 2001; Keller & Waller 2002; Packard 2003).

Both a diversity of mating mechanisms and inbreeding avoidance are facilitated by reintroduction to large protected areas where introduced populations can expand and new packs can readily be established. In contrast, populations that are more geographically constrained may allow fewer opportunities to avoid inbreeding. In captivity, inbreeding is common among wolves, suggesting that the desire to reproduce is stronger than inbreeding avoidance (Laikre & Ryman 1991; Kalinowski *et al.* 1999; Packard 2003). Similarly, the small wolf population in Isle Royale National Park, Michigan, has half the variation of mainland conspecifics, and heterozygosity has declined with every generation (Wayne *et al.* 1991; Peterson *et al.* 1998). Finnish and Swedish populations of grey wolves are small and restricted to limited areas and have lower levels of heterozygosity (Ellegren 1999; Vilà *et al.* 2003; Bensch *et al.* 2006). The Swedish population in particular suffers from inbreeding depression (Liberg *et al.* 2005; Bensch *et al.* 2006). Nonetheless, individuals in that population appeared to mate with wolves having higher levels of heterozygosity in the absence of unrelated mates (Bensch *et al.* 2006). In

comparison, we found no evidence that breeders had higher levels of heterozygosity than nonbreeders. This finding may be more typical of wolf populations such as those in Yellowstone that have low levels of inbreeding and uniformly high levels of individual heterozygosity. These high levels of genetic diversity suggest that large-scale reintroductions can better preserve variation in the short term through a variety of mechanisms, given ample high quality habitat and the opportunity for the population to expand quickly. If such conditions are not possible, artificial migration may be the best option for preserving variation. For example, a single immigrant appears to have rescued the Swedish wolf population from high rates of inbreeding and loss of heterozygosity (Mills & Allendorf 1996; Ellegren 1999; Vila *et al.* 2003; Bensch *et al.* 2006). Future research is needed to determine how factors such as wolf density, prey density, dispersal, and territoriality influence pair and pack formation, and its subsequent influence on genetic diversity.

Interpack dispersal and relatedness

Interpack dispersal was common during the study period and helped maintain the observed genetic trends of high heterozygosity and low inbreeding coefficients (Fig. 7). All genetically verified immigrants were males and all bred. Other studies have recorded the presence of adopted nonbreeding pack members (Mech & Boitani 2003a), although only one confirmed genetic relatedness of the adoptees (Lehman *et al.* 1992). We found that no females were immigrants into a pre-established pack; they were involved in pack splitting events with territories often established next to their natal pack. For example, Druid Peak female groups split to form adjacent packs of Agate Creek and Geode Creek (Fig. 4). However, single females also joined groups of males, as found in the formation of Swan Lake pack and Cougar Creek pack. In general, kinship ties were biased by proximity with the majority kinship ties existing between neighbouring packs. Conceivably, such kinship ties may promote social stability and pack persistence (Wayne 1996). However, such ties do not mitigate interpack strife in Yellowstone, as both are common between packs sharing territorial boundaries (Yellowstone Wolf Project, NPS, unpublished data), and overall rates of interpack interaction are as high in Yellowstone as elsewhere, even in areas where interpack relatedness values are low (Lehman *et al.* 1992).

Breeding scenarios and preservation of genetic variation

In general, the breeding behaviours of Yellowstone wolves resulted in preservation of genetic variation in the breeding pool that did not differ substantially from that of a managed breeding strategy (Fig. 2). To understand the implications

of different breeding strategies across one generation, we simulated a single generation of offspring for four mating schemes. These results showed that restricting potential mates to individuals within packs had a dramatic effect on genetic variability, with mean heterozygosity about 10% lower than observed. Even an open breeding strategy, which utilized the entire breeding pool, retained less variation than observed. These findings support pedigree evidence that active choice of unrelated mates within or outside of the pack structure is occurring. As expected, the managed breeding strategy preserved genetic variation most effectively overall. Strikingly, the observed population's level of heterozygosity was included in the distribution of the managed breeding strategy, indicating that the natural social behaviour of wolves is sufficient to preserve high levels of variation given access to unrelated mates and a large diverse breeding pool. Captive breeding strategies that mimic such natural breeding behaviours will only minimally reduce levels of genetic variability over a closely managed strategy and at the same time maintain natural patterns of social interactions. When feasible, captive breeding strategies should promote the natural formation of packs and diverse opportunities for pair formation, such as timely replacement of lost breeders with unrelated individuals and allowing for subordinate breeding.

Long-term concerns and conservation implications

The Endangered Species Act in the United States (USFWS 1973) requires a recovery plan for species listed as endangered. The Western grey wolf is a listed species and has a modest recovery plan that has been enacted requiring only 30 breeding pairs for three consecutive years evenly distributed among the central Idaho, GYA and northwestern Montana recovery area. This recovery goal was met in 2002 (USFWS *et al.* 2005) but is far below historical values based on genetic analysis of over 350 000 individuals (Leonard *et al.* 2005). Currently, the states of Idaho, Wyoming and Montana have prepared plans for grey wolf management after proposed federal delisting with goals of maintaining at least 10 breeding pairs and at least 100 wolves per state (USFWS 2007). Our results show that populations of this size that remain isolated will lose genetic variation and become inbred over the long term (Fig. 8). Consequently, we suggest that future management of Western wolf populations incorporate genetic data regarding population structure, minimal viable population sizes, and the degree of isolation following population reestablishment. Such information will help assess recovery success and identify areas of concern for both short- and long-term genetic viability. For Yellowstone wolves, our viability predictions suggest that a minimum population size of 170 individuals is adequate for short-term retention of genetic variability. Over the long term, however, genetic variation will decrease

and inbreeding will increase without additional migration from other populations or substantial increases in population size over this minimum value. In general, for each of the three recovery areas, an absence of gene flow will lead to decreased genetic variation and loss of the potential for recolonization in the event of population extinction (Eldridge *et al.* 1999; Frankham *et al.* 2002; Aspi *et al.* 2006; Hazlitt *et al.* 2006). However, northwestern Montana wolf populations genetically communicate with those in southern Canada (Forbes & Boyd 1997), and central Idaho currently has a large enough population size ($N > 700$) and connectivity to northwestern Montana populations to delay any immediate concerns about the loss of genetic variation.

In contrast, the YNP population appears to be genetically isolated and has reached carrying capacity at about 170 individuals. The rate of decrease in heterozygosity and increase in inbreeding over the near term (the next 20–30 years) are low enough that phenotypic signs of inbreeding depression such as skeletal defects or a significant decrease in offspring survivorship are not predicted (Hedrick *et al.* 2001; Raikonen *et al.* 2006). However, a recent study examining the effect of inbreeding depression on offspring survivorship in an inbred Swedish population (Liberg *et al.* 2005) indicates a decrease in juvenile survivorship by approximately 15% with an increase in the inbreeding coefficient of 0.1. In the Yellowstone population, we predict that the inbreeding coefficient will rise to 0.1 in approximately 60 years without gene flow from outside the park. Given these results, we would expect to observe an increase in juvenile mortality from an average of 23 to 40%, an effect equivalent to losing an additional pup in each litter. To deter such inbreeding effects, migration will be needed, involving translocation of wolves from elsewhere or the development of specific habitat corridors. The latter may be feasible if populations in the northern Rocky Mountains are genetically connected and inter-population dispersal occurs (Sunquist & Sunquist 2001; Waser *et al.* 2001). However, only low-quality corridors currently connect the GYA to the Idaho and northwestern wolf populations, exposing dispersers to high human-associated mortality risks (Oakleaf *et al.* 2006). The genetic impact of this isolation may take decades to accumulate but can be delayed if gene flow with other populations is established and maintained.

In conclusion, we show that in addition to a genetically diverse founding stock, the maintenance of genetic variation is dependent on a wide variety of behavioural mechanisms for avoiding inbreeding with close relatives. We found no natural breeding pairs that were closely related which confirmed previous results on wolves from Minnesota and Alaska (Smith *et al.* 1997). Such inbreeding avoidance was facilitated by specific population assembly patterns including avoidance of breeding with related pack

members, dispersal of males to packs where they are unrelated to the breeding females, and the fission of packs with a high proportion of close relatives to include adult offspring that are joined by dispersing and unrelated adults of the opposite sex. In general, a wide diversity of mechanisms for breeding pair formation promoted retention of genetic variability in the Yellowstone population. Simulation results showed that the observed levels of genetic variation were higher than that expected by random breeding within packs or across the entire breeding pool. This observed bias in breeding occurs despite the high probability of mating with close relatives in the Yellowstone population and results in levels of variation similar to that of a population managed for high levels of variation and reduced inbreeding. Consequently, population management should include efforts to ensure that the social dynamics function remain unhindered, thus promoting the diversity of behaviours that allow for inbreeding avoidance and pack formation as found in the Yellowstone population. These actions might include the maintenance of a high quality core habitat that will allow a rapid increase and establishment of a founder population, and genetic communication between networks of adjoining packs. In Yellowstone, kinship ties predominate between packs sharing a common territorial boundary implying that interpack dispersal is a key feature of natural populations (Lehman *et al.* 1992). Over the short term, core areas the size of YNP containing 10–12 packs appear sufficient to maintain genetic variation and may act as source populations for nearby sink regions such as the GYA where control actions occur. However, intense control actions in the region may severely affect the continuity of pack systems and hinder genetic exchange. Moreover, if such actions result in the removal of breeding pairs, this may alter the stability of pack dynamics, leading to higher breeder turnover and more frequent occurrence of inbreeding as mating choices become limited to close relatives.

Overall, our findings demonstrate the effectiveness of the reintroduction in preserving genetic diversity over the first decade of wolf recovery in Yellowstone. Our analyses suggest that little more could have been done to improve the maintenance of variation, which is a testament to both the original reintroduction design as well as the importance of having large-scale and high quality ecosystems where natural behavioural processes can be maintained. Detailed population genealogies can provide valuable insight into the dynamics influencing both genetic and social structure of reintroduced populations, and in some cases, may identify some of the causes and consequences of limitations in breeding opportunities (e.g. Liberg *et al.* 2005; Bensch *et al.* 2006). These limitations can potentially be addressed by management actions such as increased protection, habitat restoration and population augmentation.

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Bridgett vonHoldt studies evolutionary genomics, genetic mapping and domestication of wild canid populations and modern dogs. Dan Stahler is the Project Biologist of the Yellowstone Wolf Project interested in the ecology, behavior and ecosystem influence of carnivores. He is currently a doctoral student studying the behavioral and molecular ecology of Yellowstone's wolves. Doug Smith is the leader of the Yellowstone Wolf Project whose research interests include conservation biology, predator-prey relationships, population dynamics, behavior, and the role of wolves in ecosystems. Dent Earl applies computational and genetic techniques to study questions in conservation biology and population genetics theory. John Pollinger studies conservation genetics of carnivores and birds and directs UCLA's Conservation Genetics Resource Center. Robert Wayne applies molecular genetic techniques to study questions in ecology, behavior and evolution of animal.

Supplementary material

The following supplementary material is available for this article:

Table S1 Demographic input values for vortex analyses for Yellowstone National Park for 2004.

Table S2 Descriptive statistics for the total population genotyped of Yellowstone National Park.

Table S3 Descriptive statistics for the pedigree population genotyped of Yellowstone National Park.

Table S4 Predicted population size needed to limit observed heterozygosity (H_O) drop to 5% over 100 years using demographic values for the population during 2004.

Table S5 Predicted effect of annual immigration for YNP on maintenance of heterozygosity in a static population ($N = 170$) over the duration of 100 years.

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