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Pedigree-based assignment tests for reversing coyote (*Canis latrans*) introgression into the wild red wolf (*Canis rufus*) population

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Abstract

The principal threat to the persistence of the endangered red wolf (*Canis rufus*) in the wild is hybridization with the coyote (*Canis latrans*). To facilitate identification and removal of hybrids, assignment tests are developed which use genotype data to estimate identity as coyote, 1/4, 1/2, 3/4 or full red wolf. The tests use genotypes from the red wolves that founded the surviving population and the resulting pedigree, rather than a contemporary red wolf sample. The tests are evaluated by analysing both captive red wolves at 18 microsatellite loci, and data simulated under a highly parameterized, biologically reasonable model. The accuracy of assignment rates are generally high, with over 95% of known red wolves identified correctly. There are, however, tradeoffs between ambiguous assignments and misassignments, and between misidentifying red wolves as hybrids and hybrids as red wolves. These result in a compromise between limiting introgression and avoiding demographic losses. The management priorities and level of introgression determine the combination of test and removal strategy that best balances these tradeoffs. Ultimately, we conclude that the use of the assignment tests has the capacity to arrest and reverse introgression. To our knowledge, the presented approach is novel in that it accounts for genetic drift when the genotypes under analysis are temporally separated from the reference populations to which they are being assigned. These methods may be valuable in cases where reference databases for small populations have aged substantially, pedigree information is available or data are generated from historical samples.

Keywords: assignment test, hybridization, likelihood ratio, microsatellite, Monte Carlo simulation, red wolf

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Introduction

Prior to European settlement, the red wolf (*Canis rufus*) occupied most of the eastern and southeastern portions of the United States (Nowak 1979; Nowak 2002). Like the grey wolf (*Canis lupus*), it was exterminated aggressively by settlers. In the last century, the coyote (*Canis latrans*) expanded its range into the southeast as a result of habitat modification and its superior ability to evade the persecutions of man (Parker 1995). By the middle of the 20th century few red wolf populations remained, and those that did were at risk due to hybridization with coyotes (McCarley 1962). In 1967 the red wolf was among the first species listed under the law that now is the Endangered Species Act. To prevent extinction, the last known wild red wolves were captured during the 1970s from a swamp along the Texas–Louisiana border. After extensive phenotypic evaluation of 400 captured individuals, 17 were selected to initiate a captive breeding program (U.S. Fish and Wildlife Service 1989) and 14 produced offspring successfully.

In 1987, red wolves were released from the captive population into Alligator River National Wildlife Refuge in North Carolina, a peninsula believed to be coyote free (Parker 1986). Although the red wolf population expanded, coyotes concurrently established themselves in adjacent areas. By the early to mid-1990s, hybridization between the two species was suspected and later

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confirmed (Kelly *et al*. 1999). The swamping of the red wolf gene pool emerged quickly as a serious threat to the continued existence of the species (Kelly *et al*. 1999). The current mandate of the red wolf recovery programme is to arrest the introgression of coyote genes into the wild red wolf population while continuing to build the red wolf population across a 1.7-million-acre experimental population area (Kelly 2000). Genetic methods to detect introgression are greatly needed.

Several genetic assignment methods have been developed which infer the ancestry of an individual from a genetic sample (Paetkau *et al*. 1995; Rannala & Mountain 1997; Blott *et al*. 1999; Cornuet *et al*. 1999; Banks & Eichert 2000; Pritchard *et al*. 2000; Hansen *et al*. 2001; Vázquez-Domínguez *et al*. 2001; Anderson & Thompson 2002; Wilson & Rannala 2003). The original assignment method was proposed by Paetkau *et al*. (1995) in which the samples of a geographical area are used to estimate the allele frequencies of that population. Each sample is assigned to the population from which the sample's multilocus genotype has the highest expected frequency. The assignment test was recast in a formal likelihood ratio framework by Rannala & Mountain (1997). In the simplest case, an individual is hypothesized to have originated either in the population from which it was sampled (the null hypothesis) or from a second population. The two hypotheses are resolved by computing the ratio of the probability of the data under each and comparing this to the ratio's distribution when the null is true. Cornuet *et al*. (1999) noted that the population of origin may sometimes be unsampled. They therefore suggested an exclusion test where all populations within which the genotype is sufficiently improbable are excluded. Pritchard *et al*. (2000) introduced a fully Bayesian method in which individual genotypes are clustered into groups which minimize deviations from Hardy–Weinberg proportions rather than defined a priori. Recently, Anderson & Thompson (2002) and Wilson & Rannala (2003) have developed other Bayesian approaches to the assignment test. The methods of Rannala & Mountain (1997), Pritchard *et al*. (2000), Anderson & Thompson (2002) and Wilson & Rannala (2003) are especially pertinent to the issue of hybridization because they consider the possibility that an individual is from neither population A nor B, but has recent ancestry in both (e.g. Randi & Lucchini 2002).

Among the central challenges in developing an assignment algorithm is the problem of defining the allele frequencies in the parental generation and the breeding structure. Methods differ in how they estimate allele frequencies, but they generally assume random mating (but see Wilson & Rannala 2003), random sampling, and that the sample represents the total available information on allele frequencies (also see Cornuet *et al*. 1999). The red wolf/coyote situation differs in that the best source of information on the red wolf allele frequencies comes from the founders and the known genealogy. All red wolves are descended from 14 founder individuals and the pedigree from the founders to the 81 red wolves released into North Carolina is known. Within the wild population, some of the pedigree is also known. By characterizing the genotypes of the 14 founder individuals, much can be inferred about allele frequencies. An even better alternative would be to use genotypes from the 81 released individuals, but unfortunately samples are not available from many of these individuals.

One objective of this study is to develop three related assignment tests which assign wild-caught individuals a category of ancestry based on their multilocus genotypes. Often these wild-caught canids are suspected hybrids or litter-mates and are therefore not random draws from the population. By using the founder genotypes and the pedigree it is possible to avoid some random sampling assumptions as well as improve estimates of allele frequencies and sampling distributions. The detailed pedigree and a wealth of field data also provide a rare opportunity to investigate the performance of the tests. The second endeavour of this paper is to evaluate the frequency with which the tests make correct, ambiguous and incorrect assignments by analysing captive red wolves and by simulating data under a biologically reasonable model. The potential effectiveness of the tests is demonstrated by applying them to hypothetical populations with differing degrees of introgression. Finally, the broader applicability of the developed methods is discussed.

Materials and methods

DNA extraction

Whole blood samples from the red wolf captive breeding programme (n = 49) were obtained and extracted using a phenol/chloroform protocol (Vardenplas *et al*. 1984). Coyote tissue samples stored in lysis buffer (Longmire *et al*. 1991) from North Carolina (NC; n = 22) and Virginia (VA; n = 30) were provided by P. Wilson (Wildlife Forensics Laboratory), Martin Lowney and Chad Fox (Wildlife Services, VA) and extracted using a QIAmp™ tissue kit (Qiagen, Valencia, CA, USA). Bone samples from the 14 red wolf founders were collected from the Slater Museum of Natural History at the University of Puget Sound and the Burke Museum of Natural History at the University of Washington. Bone samples were extracted using the silica extraction method (Boom *et al*. 1990; Höss & Pääbo 1993). To avoid cross-contamination, bone extractions occurred in a separate building where no forms of concentrated canid DNA were allowed. All extractions were performed with at least one negative control to monitor for contamination.

Microsatellite loci

Nineteen nuclear microsatellite loci were selected from among the 30 tested preliminarily, based on consistency of amplification, ease of scoring and variability: AHT103, AHT121 (Holmes *et al*. 1995), CXX250, CXX377, CXX2001, CXX2004, CXX2010, CXX2054, CXX2062, CXX2145 (Mellersh *et al*. 1997), CXX172, CXX225, CXX30, CXX204, CXX109, CXX173, CXX250, CXX20 (Ostrander *et al*. 1993) and CXX403 (Ostrander *et al*. 1995). The 19 loci were multiplexed into nine PCR reactions. The 15 μL reaction mix for multiplex 1 consisted of 0.2 μM primer 377 and 204, 0.4 μM primer 109, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 1× Gold *Taq* DNA polymerase buffer supplied by the manufacturer (Applied Biosystems), 1.5 μL of DNA extract and 0.5 U of Gold*taq* DNA polymerase (Applied Biosystems). All other polymerase chain reactions (PCR) contained the same mixture with differing amounts of primers and $MgCl_2$. The PCR mix for multiplex 2 contained 0.2 μM primer 172 and 173 and 0.6 μM 200 with 1.5 mM $MgCl_2$. The PCR mix for multiplex 3 contained 0.2 μM primer 2004, 30 and 2145, with 1.0 mM $MgCl_2$. The PCR profile for multiplexes 1–3 was 55 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, after an initial denaturation step of 95 °C for 10 min. All other PCR profiles differ only by annealing temperatures. The PCR mix for multiplex 4 contained 0.13 μM primer 103 and 0.2 μM primer 20 with 1.5 mM $MgCl_2$. The PCR mix for multiplex 5 contained 0.1 μM 2001 and 0.2 μM 403, with 1.0 mM $MgCl_2$. The PCR mix for singleplex 6 contained 0.2 μM primer 121 with 2.0 mM $MgCl_2$. The PCR mix for singleplex 7 contained 0.2 μM primer 250 with 2.0 mM $MgCl_2$. The PCR profile for multiplexes 4–7 had an annealing temperature of 55 °C. Multiplex 8 contained 0.2 μM primer 2062 and 225 and 0.6 μM primer 2010, with 2.0 mM $MgCl_2$, and an annealing temperature of 53 °C. Singleplex 9 contained 0.2 μM primer 2054, with 1.0 mM $MgCl_2$, and an annealing temperature of 63 °C. All PCRs contained a positive and negative control. All samples were run on a 377 ABI Automated Sequencer following manufacturers' protocols. Allele sizes were scored using GENESCAN and GENOTYPER 2.5 (Perkin-Elmer).

Assignment tests

All three of the tests presented here make the assumption that canids in the experimental population area belong to one of six categories of identity: 100% red wolf (RW), 100% coyote (Coy), $^3/_4$RW (RW × F_1), $^1/_2$RW [composed of F_1 (RW × Coy) and $F_2(F_1 \times F_1)$], or $^1/_4$RW (Coy × F_1). We define as pure red wolf the 14 founders and any individual descended solely from them. We do not address any possible hybridization between red wolves and coyotes prior to red wolves being brought into captivity (McCarley 1962) or the putative hybrid origin of the red wolf (Wayne & Jenks 1991; Roy *et al*. 1994; Roy *et al*. 1996).

Coyote private allele (CPA) test. A coyote private allele (CPA) is defined as any allele with frequency greater than zero in the coyote allele distribution and frequency zero in the red wolf distribution (see next section on likelihood ratio test for details on allele frequencies). The number of CPAs is counted for the genotype under analysis. For each of the six identities the distribution of CPAs is generated by simulation. The number of CPAs is located in the distribution for each identity; if it falls in the tail of the distribution the identity is rejected. Otherwise it remains a plausible identity.

The CPA distributions are generated according to the following four steps:

(i) *Simulate coyote allele frequencies*. To account for sampling error, coyote allele frequencies are obtained by bootstrap resampling from the observed coyote allele frequencies until the real sample size is obtained ($2n = 104$). Because effective population size in coyotes is large and genetic drift therefore very weak, simulated frequencies are assumed not to change over time.

(ii) *Simulate red wolf allele frequencies*. Red wolf allele frequencies are simulated in two steps corresponding to captive and wild phases. In the captive phase, alleles pass from founders through descendants to the released wolves assuming Mendelian segregation (i.e. each allele has a $^1/_2$ chance of being passed to each offspring). Allele frequencies among the released individuals are calculated from their simulated genotypes. In the wild phase, drift occurs under the basic Wright–Fisher model (Gillespie 1998) for four generations with $N_e = 10$ (Fig. 1). Four generations should represent a conservatively large amount of drift as 1993 is the mean year of release and a generation in red wolves is ~4 years. The third and fourth generations in the wild are denoted *t*-2 and *t*-1, respectively. The known aspects of the wild pedigree are therefore ignored in generating sampling distributions.

In both the captive and wild phases, each allele at each locus has an independent 0.001 probability of mutating each generation (Goldstein & Pollock 1997). When a mutation occurs, it is stepwise with probability 0.9. If it is stepwise, a gain or loss of one step is assumed equally probable unless the allele is already at the boundary, in which case it mutates one step inward. The boundaries at each locus are defined as one step beyond the shortest and longest alleles observed in the combined set of coyote and red wolf alleles. If it is not a stepwise mutation, it mutates to a randomly drawn allele greater than one step away. The set of possible alleles includes all steps between the boundaries.

(iii) *Simulate genotypes*. Red wolf genotypes are simulated by drawing two alleles at each locus at random and with replacement from the *t*-1 allele frequencies (Fig. 1). Coyotes are generated from the simulated coyote frequencies. To simulate F_1s, one *t*-1 red wolf and one coyote allele are

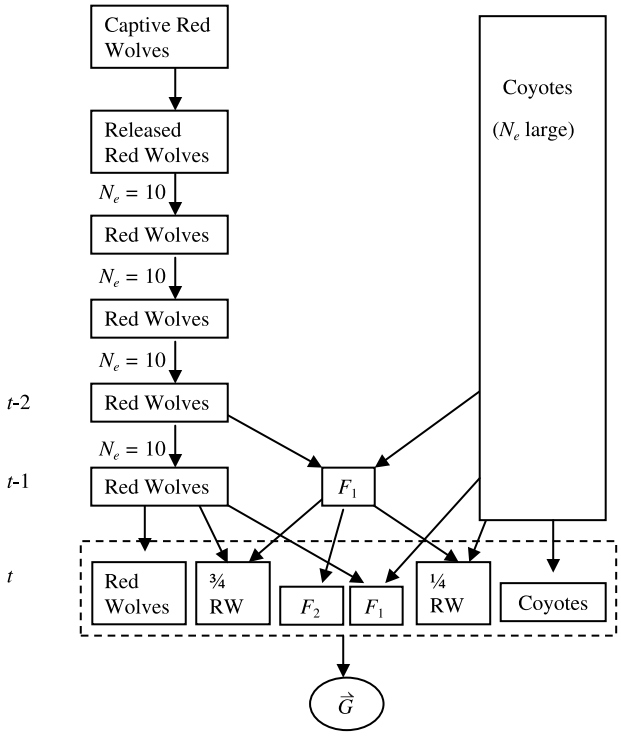

Fig. 1 Model used to simulate genotypes ($\vec{G}$) from which CPA and LR sampling distributions are derived. The sole disparity between this model and that used to calculate the likelihood of $\vec{G}$ is that no drift (i.e. a large N_e in the red wolf) is assumed in calculating the likelihood. Pool of sampled genotypes is temporally indexed by *t* while *t*-1 and *t*-2 indicate the parental and grandparental generations (see text). Note that this is not the model used to simulate genotypes for analysis; that model uses the known pedigree and is considerably more complex.

drawn at each locus. F_2s are created by simulating two F_1s from the *t*-2 red wolf and coyote frequencies and then drawing a random allele from each. To simulate $^3/_4$RWs, a F_1 is created from the *t*-2 red wolf and coyote frequencies, a red wolf is created from the *t*-2 frequencies and a random allele is drawn from each parent. Finally, $^1/_4$RWs are generated by creating an F_1 from the *t*-2 and coyote frequencies, creating a coyote and then drawing a random allele from each.

(iv) *Create distributions*. For each of the six identities 250 individuals are generated, scored for CPAs and sorted to form a cumulative CPA distribution. Notice that this distribution depends on the particular allele frequencies simulated in steps (i) and (ii). Another pair of simulated populations would produce a slightly different set of CPA distributions. This problem is addressed by repeating steps (i) to (iii) 250 times. Each time, for each identity a CPA distribution is generated.

When analysing a real genotype, the *P*-value is calculated under each identity for each of the 250 replicate distributions by finding the proportion of the CPA distribution ≤ the observed number of CPAs, the proportion ≥ the observed number of CPAs, and taking the smaller of these values. A one-tailed test is appropriate because our primary objective in the CPA test is separating red wolves from hybrids. If the observed number of CPAs is smaller than the smallest value in the distribution or greater than the largest value, then a *P*-value of 1/250 (0.004) is assigned. Thus, analysing a sample yields 250 *P*-values for each identity. Sorting produces six *P*-value distributions. The predefined κ quantile then defines the *P*-value for each identity. The plausible identities are those with *P*-values greater than α. A sequential bonferroni correction is employed (Rice 1989). In the very rare event that all six identities are rejected, the identity with the largest *P*-value is assigned. The appropriate value of κ was determined in preliminary simulations (using methods described in 'evaluating accuracy of assignment tests') by finding the value for κ, where the rate at which the true identity of a simulated individual is falsely rejected (type I error) ≈ α (the test size). Although a different summary statistic is used and distributions are generated in a way tailored to the situation, the CPA test is analogous to the exclusion test of Cornuet *et al*. (1999).

Likelihood ratio (LR) test

The likelihood ratio (LR) test utilizes the differences in allele frequencies between red wolves and coyotes. For calculating likelihoods, the coyote allele frequencies are those observed in a sample of 52 coyotes from neighbouring regions of NC and VA. These areas are assumed to be far enough away to be virtually unaffected by hybridization in the experimental population area but close enough to have frequencies similar to the coyotes on and adjacent to the refuge. To confirm that pooling the two coyote samples is justified and that they are very similar to those near the experimental population area a test for allele frequency homogeneity using GENEPOP on the web (Raymond & Rousset 1995) is conducted. The two coyote populations are also tested for Hardy–Weinberg proportions. The test size (α) is set to 0.05 and a sequential bonferroni correction is applied (Rice 1989). F_{ST} (θ) is estimated between the two coyote samples and between the red wolf founders and the pooled coyote sample using GENEPOP (Weir & Cockerham 1984).

The red wolf frequencies represent the expected frequencies of alleles among the 81 released individuals. These are derived by genotyping 13 of the 14 founders plus the three offspring of the 14th (no material was available from the 14th). Throughout this study, these 16 are referred to as founders. Using the known pedigree (obtained from William Waddell, Red Wolf SSP Coordinator), the proportional contribution of each founder to the 81 released wolves is calculated. The expected frequency of each allele at each locus is obtained by weighting the frequency of the

allele within each founder (0, 0.5 or 1) by the founder's contribution summed over founders.

In the LR test, the likelihood of the genotype under each of the six putative identities is calculated. The identity under which the likelihood is largest is termed the likelihood maximizing identity (LMI). To determine which of the other five identities are also plausible explanations of the data, each of them is considered a null hypothesis. The ratio of the likelihood of the LMI hypothesis to the null hypothesis is calculated and then located in the distribution of the ratio when the null is true. If the calculated ratio falls in the tail the null identity is rejected, otherwise it is included among the unrejected identities along with the LMI.

The likelihood of the genotype is calculated by making several assumptions: estimated allele frequencies are the parametric frequencies, frequencies do not change between generations, loci are independent and breeding is random within a particular cross of identities (e.g. RW × RW). Let the multilocus genotype be designated $\vec{G}$. Let M and H denote the number of homozygous and heterozygous loci, individually identified by the subscripts i and j, respectively. If an unknown canid is homozygous at locus i let the frequency of the observed allele be p_i in the red wolf and p_i' in the coyote. If the canid is heterozygous at locus j, let the frequencies of the two alleles be p_j and q_j in the red wolf and p_j' and q_j' in the coyote. The probability of $\vec{G}$ under each identity is then given by the following equations.

$$P(\vec{G} \mid \mathrm{RW}) = \prod_{i=1}^{M} p_i^2 \quad \prod_{j=1}^{H} 2p_jq_j \tag{1}$$

$$P(\vec{G} \mid \mathrm{Coy}) = \prod_{i=1}^{M} p_i'^2 \quad \prod_{j=1}^{H} 2p_j'q_j' \tag{2}$$

$$P(\vec{G} \mid F_1) = \prod_{i=1}^{M} p_ip_i' \quad \prod_{j=1}^{H} (p_jq_j' + p_j'q_j) \tag{3}$$

$$P(\vec{G} \mid \mathrm{RW} \times F_1) = \prod_{i=1}^{M} p_i \left(\frac{p_i + p_i'}{2} \right) \prod_{j=1}^{H} p_jq_j + \left(\frac{p_jq_j' + p_j'q_j}{2} \right) \tag{4}$$

$$P(\vec{G} \mid \mathrm{Coy} \times F_1) = \prod_{i=1}^{M} p_i' \left(\frac{p_i + p_i'}{2} \right) \prod_{j=1}^{H} p_j'q_j' + \left(\frac{p_jq_j' + p_j'q_j}{2} \right) \tag{5}$$

$$P(\vec{G} \mid F_1 \times F_1) = \prod_{i=1}^{M} \left(\frac{p_i + p_i'}{2} \right)^2 \prod_{j=1}^{H} 2 \left(\frac{p_j + p_j'}{2} \right) \left(\frac{q_j + q_j'}{2} \right) \tag{6}$$

When an allele has frequency zero in either the coyote or red wolf distributions it makes some of the likelihoods zero. In the very rare event that an allele is encountered that has a frequency of zero in coyotes (red wolves have very few private alleles), it is reassigned a frequency of $1/(2n + 1)$ (i.e. 1/105; Paetkau *et al*. 1995). An allele observed in recent red wolves that is not observed in the founders can have arisen only by mutation. Assuming seven generations have transpired since the founders, and that each allele has a 0.001 probability of mutating to a new allele each generation, then the probability that a random allele has mutated to a new allele is $\approx 0.007\ [1 - (1 - 0.001)^7]$. We ignore the fact that many mutations would not have produced a new allele and reassign an allele with zero frequency in red wolves a frequency of 0.007. We acknowledge that this should be an overestimate of the expected frequency of this allele in the red wolf.

To determine which of the five non-LMI identities are excluded we treat each as a null and calculate $\Lambda = \ln(\vec{G} \mid \textit{null identity}) - \ln(\vec{G} \mid \mathrm{LMI})$. The value of Λ is located in the distribution of Λ when null identity is true. The distributions under the null are based on individuals of known identity simulated using exactly the same algorithm employed in the CPA test (steps i, ii, and iii). The final step (iv) of generating the distributions differs from the CPA test:

(iv) *Create LR distributions*: 250 individuals of each identity are created from simulated coyote and red wolf populations. The difference in the log-likelihood under the true identity and each of the five other untrue identities is calculated: $\Lambda_{simulated} = \ln(\vec{G} \mid \textit{true identity}) - \ln(\vec{G} \mid \textit{untrue identity})$. This yields 250 $\Lambda_{simulated}$ values associated with each of the five untrue identities for each true identity. They are sorted in ascending order to provide five cumulative distributions. The process is repeated for 250 simulated pairs of populations to yield 5×250 distributions for each true identity.

In analysing a real genotype, the comparison of the LMI to each of the five null identities results in 250 *P*-values. As in the CPA test, they are sorted into a distribution of *P*-values. The *P*-values defining the κ quantile are obtained and compared to α after Bonferroni correction for multiple tests (Rice 1989). Null identities for which the *P*-value is $\leq$ the adjusted α are rejected as inconsistent with the data. As with the CPA test, the optimal value for κ was determined by preliminary simulations. The LR test is modelled after the methods of Rannala & Mountain (1997) and Banks & Eichert (2000).

Mixed test

The mixed test represents an *ad hoc* decision rule for combining results from the first two tests. In the mixed test, if the CPA test classifies an individual as unambiguously RW then classify the individual as RW irrespective of LR test output; otherwise follow the LR test classification. The mixed test is based on observations from preliminary simulations that pure red wolves almost never carry any CPAs while $^3/_4$RWs nearly always do. The mixed test

combines the resolving power of the CPA test at the RW/non-RW boundary with the overall power of the LR test.

Evaluating the accuracy of the assignment tests

We used real and simulated individuals of known identity to evaluate the assignment accuracy of the CSA, LR and mixed tests. The real individuals consisted of 35 captive red wolves born between 1986 and 1999. The simulated individuals were generated through a highly parameterized, individual-based model. The objective was to produce a large number of genotypes of various ancestries which reflect biological realities such as overlapping generations, high variance in reproductive success, pair-bonds and grades of introgression beyond $^1/_4$, $^1/_2$ and $^3/_4$. The simulations were built upon the known pedigree from captive and wild animals. Genotypes of released red wolves were simulated by dropping alleles through the pedigree in a Mendelian manner. The mutational model described in the CPA test section was applied to all reproductive events in the simulation. Simulation of the wild canid population began in 1987 when red wolves were released and proceeded year by year according to an algorithm that uses reasonable levels of extra-pedigree pairings, litter sizes, survival and hybridization. Details of the algorithm are given in Appendix I.

All individuals born in the intervals 1998–2002 and 2008–11 were analysed using all three tests with $\alpha = 0.1$ and $\kappa = 0.5$ and 0.9. For each combination of test and κ, each individual was binned into one of 8 assignment categories: RW, {RW, $^3/_4$RW}, {RW, $^3/_4$RW, $\leq ^1/_2$RW}, $^3/_4$RW, {$^3/_4$RW, $^1/_2$RW}, {$^3/_4$RW, $^1/_2$RW, $\leq ^1/_4$RW}, $^1/_2$RW and Sum < $^1/_2$RW (which includes {$^1/_2$RW, $^1/_4$RW}, {$^1/_2$RW, $^1/_4$RW, Coy}, $^1/_4$RW, {$^1/_4$RW, Coy}, and Coy). The $^1/_2$RW identity was used to simplify results and represents assignment as F_1 and/or F_2. Individuals were divided among 13 true identity categories, which range from red wolf to coyote. By summing all observations within each of the 13 true identity categories, the counts were converted to estimated assignment rates for each run and over all runs. One hundred global simulations were conducted. Point estimates are all based on means across all 100 runs. For certain identities where the number of observations per run was > 15 (i.e. for RW, $^3/_4$RW and $^1/_2$RW) the 100 observed rates for each assignment bin were sorted to obtain lower and upper 5% bounds.

In order to quantify the combined effects of incorporating the known pedigree, sampling error and genetic drift, the same simulated data are re-analysed using distributions that do not incorporate these phenomena. This was done by skipping steps (i) and (ii) (see section on CPA test) and simulating genotypes (step iii) directly from the observed coyote allele frequencies without resampling and from the founder red wolf allele frequencies without incorporating drift or mutation.

Evaluating short-term impact of tests

The efficiency of the various tests for controlling introgression is explored by comparing the levels of introgression in hypothetical populations before and then immediately after a single filtering event with the proposed assignment tests. Two hypothetical populations are considered: low introgression (80% RW, 5% $^7/_8$RW, 5% $^3/_4$RW, 5% $^5/_8$RW and 5% $^1/_2$RW = 93.75% RW genepool) and high introgression (40% RW, 15% $^7/_8$RW, 15% $^3/_4$RW, 15% $^5/_8$RW and 15% $^1/_2$RW = 81.25% RW red wolf). The population is sampled either at an intensity of 50% or 100%. At 50%, exactly half of each identity is sampled (i.e. sampling is deterministic and unbiased). The sampled population is then analysed under each test. For each, the mean assignment rates for each identity are multiplied by the proportion of the (sampled) population of that identity (i.e. the analysis is assumed to deterministically follow the mean assignment rates). This yields the proportion of the population in each assignment bin. The assigned sample is then subject to one of three removal criteria (RC) describing who is removed and who is returned to the population.

RC 1: only canids assigned RW are returned and all others are removed.
RC 2: only individuals assigned RW or {RW, $^3/_4$RW} are returned.
RC 3: only individuals assigned RW, {RW, $^3/_4$RW} or $^3/_4$RW are returned.

Note that throughout this study identities enclosed within brackets, {}, and separated by a comma represent a list of unexcluded identities.

The impact is measured by percentage reduction in the total population along with the proportional contribution of red wolves to the postremoval genepool. For comparison, the impact of each RC in combination with a perfect test where all true identities are known exactly and without error is calculated.

Results

Tests for Hardy–Weinberg (HW) proportions revealed that locus 204 has a severe and highly significant excess of homozygotes ($P < 0.0001$) in the VA coyote population. Locus 204 also shows significant differentiation between populations ($P < 0.0001$). The locus was removed from the data set leaving 18 loci. Locus 30 also deviates significantly ($P < 0.0001$) from HW proportions in the VA population, although there is no evidence of population differentiation ($P = 0.20$). Observations from the VA population are excluded

Table 1 Assignment breakdown by count (and percentage) of 35 captive red wolves by test. In all tests $\alpha = 0.1$

	Assignment (unexcluded identities)	
Test	RW	RW, $^{3}/_{4}$RW
CPA	35 (100)	0 (0)
LR	34 (97)	1 (3)
Mixed	35 (100)	0 (0)

and the NC samples are used to estimate allele frequencies at the locus. Among other loci there are no significant deviations from HW proportions within populations nor any significant tests for population differentiation. F_{ST} between VA and NC is estimated at 0.01. These results justify pooling the samples to estimate allele frequencies. They also support the premise that allele frequencies in VA and NC are representative of frequencies among coyotes on the experimental population area. F_{ST} between the founder red wolves and the pooled coyote samples is estimated at 0.13. The mean number of CPAs per locus is 4.7. Within coyotes, the average locus is composed of 30.7% CPAs. A total of two red wolf private alleles (0.1 per locus) was observed.

The value of κ which results in type I errors $\approx \alpha$ varies somewhat between tests and between identities. In the CPA test, $\kappa = 0.5$ was found to be sufficient in limiting type I errors to $\leq \alpha$ (results not shown). In the LR test, $\kappa = 0.5$ limited type I errors to $\leq \alpha$ in RWs, $^{1}/_{2}$RWs (F_1s and F_2s) and Coys, but $\kappa \approx 0.9$ was necessary for $^{3}/_{4}$ and $^{1}/_{4}$RWs. For the duration of this study we set $\kappa = 0.5$. This is done for the sake of simplicity and because even in the LR test, $\kappa = 0.5$ limits the specific assignment errors of greatest concern here (e.g. RW assigned non-RW, $^{3}/_{4}$RW assigned RW) to approximately α or smaller.

The 35 captive red wolves are classified as red wolves with a high degree of accuracy in all three tests (97–100% with $\alpha = 0.1$; Table 1). The LR test is slightly conservative, classifying one red wolf as {RW, $^{3}/_{4}$RW}. The observed assignment rates for the 1998–2002 simulated data with α

Table 2 Observed mean assignments percentages for simulated individuals born between 1998 and 2002 by test. In parentheses are lower and upper 5% rates among 100 runs. Mean number of observations per run is given in last row of table. 'Sum < $^{1}/_{2}$RW' assignment bin is sum of several more specific assignments bins: {$^{1}/_{2}$RW, $^{1}/_{4}$RW}, {$^{1}/_{2}$RW, $^{1}/_{4}$RW, Coy}, $^{1}/_{4}$RW, {$^{1}/_{4}$RW, Coy} and Coy. Assignment identity $^{1}/_{2}$RW represents assignment as F_1 and/or F_2

		True identity by proportion RW												
Test	Assignment (unexcluded identities)	1 = RW	< 1 > $^{7}/_{8}$	$^{7}/_{8}$	< $^{7}/_{8}$ > $^{3}/_{4}$	$^{3}/_{4}$	< $^{3}/_{4}$ > $^{5}/_{8}$	$^{5}/_{8}$	< $^{5}/_{8}$ > $^{1}/_{2}$	$^{1}/_{2}$	< $^{1}/_{2}$ > $^{1}/_{4}$	$^{1}/_{4}$	< $^{1}/_{4}$ > 0	0 = Coy
CPA	RW	94 (79, 100)	46	17	8	3 (0, 11)	1	1						
$\alpha = 0.1$	RW, $^{3}/_{4}$													
	RW, $^{3}/_{4}$, $\leq ^{1}/_{2}$													
	$^{3}/_{4}$RW	6 (0, 21)	49	62	53	34 (13, 61)	25	12	11	2 (0, 11)	1			
	$^{3}/_{4}$RW, $^{1}/_{2}$		3	14	19	26 (12, 39)	25	20	8	7 (0, 17)	3	3		
	$^{3}/_{4}$RW, $^{1}/_{2}$, $\leq ^{1}/_{4}$		2	7	19	35 (10, 63)	43	53	51	54 (36, 72)	34	25	15	2
	$^{1}/_{2}$RW													
	Sum < $^{1}/_{2}$RW				1	2 (0, 8)	6	14	27	37 (20, 57)	62	72	85	98
LR	RW	95 (79, 100)	70	39	22	8 (0, 22)	2	2						
$\alpha = 0.1$	RW, $^{3}/_{4}$	4 (0, 13)	14	17	17	7 (0, 18)	4	3	1					
	RW, $^{3}/_{4}$, $\leq ^{1}/_{2}$													
	$^{3}/_{4}$RW	1 (0, 9)	14	35	44	54 (31, 72)	41	22	11	3 (0, 12)	1			
	$^{3}/_{4}$RW, $^{1}/_{2}$	0 (0, 3)	1	6	9	17 (3, 33)	21	17	9	9 (0, 18)	4	1		
	$^{3}/_{4}$RW, $^{1}/_{2}$, $\leq ^{1}/_{4}$													
	$^{1}/_{2}$RW		1	3	8	14 (0, 31)	30	46	64	73 (55, 88)	42	16	1	
	Sum < $^{1}/_{2}$RW					0 (0, 3)	2	10	15	15 (0, 32)	53	83	99	100
Mixed	RW	99 (94, 100)	73	42	24	9 (0, 27)	3	2						
$\alpha = 0.1$	RW, $^{3}/_{4}$	1 (0, 4)	12	15	15	6 (0, 17)	4	3	1					
	RW, $^{3}/_{4}$, $\leq ^{1}/_{2}$													
	$^{3}/_{4}$RW	0 (0, 3)	13	34	44	53 (30, 72)	40	22	11	3 (0, 12)	1			
	$^{3}/_{4}$RW, $^{1}/_{2}$		1	6	9	17 (3, 33)	20	17	9	9 (0, 17)	4	1		
	$^{3}/_{4}$RW, $^{1}/_{2}$, $\leq ^{1}/_{4}$													
	$^{1}/_{2}$RW		1	3	8	14 (0, 31)	31	46	64	73 (55, 88)	42	16	1	
	Sum < $^{1}/_{2}$RW					1 (0, 3)	2	10	15	15 (0, 32)	53	83	99	100
	$\bar{n}$	53	7	16	4	32	4	2	1	38	11	5	1	17

Table 3 Important subset of observed assignment rates using the mixed test under differing analysis conditions. Analysis (a) is identical to that shown in Table 2 and is included here for reference. For simplicity only three true identities are shown. 'Sum < $^{3}/_{4}$RW' assignment bin is sum of several more specific assignments bins: {$^{3}/_{4}$RW, $^{1}/_{2}$RW}, {$^{3}/_{4}$RW, $^{1}/_{2}$RW, $^{1}/_{4}$RW}, {$^{1}/_{2}$RW, $^{1}/_{4}$RW}, {$^{1}/_{2}$RW, $^{1}/_{4}$RW, Coy}, $^{1}/_{4}$RW, {$^{1}/_{4}$RW, Coy} and Coy

	Analysis description			Assignment (unexcluded identities)	True identity by proportion RW		
	α	Dists. assume drift & mutation?	Years sampled		1 = RW	$^{7}/_{8}$	$^{3}/_{4}$
(a)	0.1	Yes	1998–2002	RW	99	42	9
				RW, $^{3}/_{4}$	1	15	6
				$^{3}/_{4}$RW		34	53
				Sum < $^{3}/_{4}$RW		9	32
(b)	0.05	Yes	1998–2002	RW	87	25	4
				RW, $^{3}/_{4}$	11	31	11
				$^{3}/_{4}$RW	1	28	37
				Sum < $^{3}/_{4}$RW	1	16	48
(c)	0.1	Yes	2008–2011	RW	99	34	13
				RW, $^{3}/_{4}$	1	19	10
				$^{3}/_{4}$RW		33	44
				Sum < $^{3}/_{4}$RW		14	33
(d)	0.1	No	1998–2002	RW	91	35	8
				RW, $^{3}/_{4}$	6	18	8
				$^{3}/_{4}$RW	2	34	46
				Sum < $^{3}/_{4}$RW	1	13	38

= 0.1 are shown in Table 2. When the true identity is red wolf, the mixed test has the highest correct assignment rate (mean = 99%) followed by the LR test (95%) and the CPA test (94%). For hybrids between RW and $^{3}/_{4}$RW, the CPA test tends toward the $^{3}/_{4}$RW assignment while the LR test and to an even greater extent the mixed test, tend toward the RW assignment. With $^{3}/_{4}$RWs the mixed and LR tests make the correct $^{3}/_{4}$RW assignment more frequently than the CPA test, but they also fail to exclude RW more frequently. The lower and upper 5% values indicate that there is substantial variability in observed rates between runs.

The most important subset of assignment rates for the same data analysed using the mixed test with α lowered from 0.1 to 0.05 is shown in Table 3b. As expected, the effect of reducing α is to increase the frequency of ambiguous assignments but decrease the rate of misassignments. Because the tests are much more accurate at identifying red wolves when α = 0.1, this value is used in most subsequent analyses. Although sample size is small, the assignment rates in 2008–11 are similar to those in 1998–2002 (Table 3c). Thus, the resolving power should not decline significantly in the near future. When the 1998–2002 simulated genotypes are re-analysed using distributions that do not incorporate the pedigree, drift or sampling error, both assignment errors and ambiguous assignments increase in frequency (Table 3d).

The exercise exploring the impacts of the tests in concert with various removal criteria across a single removal event illustrates that introgression is controllable, but the greater the extent of control, the greater the demographic cost (Table 4). For both low and high levels of preremoval introgression, when the less aggressive removal strategies 2 or 3 are pursued, the mixed test is generally best in achieving the target level of postremoval introgression and limiting demographic loss. When a more aggressive removal strategy is pursued (RC 1), the CPA test performs slightly better at reducing introgression, but it also carries significantly higher demographic costs. Irrespective of preremoval introgression levels or removal strategy, the mixed test is always as good as or better than the LR test.

Discussion

Controlling introgression in the red wolf

Two of the main objectives of the red wolf recovery programme are to minimize or prevent introgression and to build and expand the population. Limiting introgression, however, comes at a demographic cost. Some of this loss reflects removals of introgressed animals that have been correctly identified by an assignment test. However, there are also assignment errors that cause removal of canids we wish to leave in the population — red wolves and perhaps individuals that are only slightly introgressed. The ideal assignment test would correctly identify hybrids a high proportion of the time while very rarely assigning red wolves (or nearly red wolves) a hybrid identity.

The problem is that these two attributes of the ideal test do not coexist. The first reason for this is that statistical tests always face a tradeoff between type I and type II errors. In

Table 4 Level of postremoval introgression and population decline as a function of preremoval introgression in population, removal criteria (RC), sampling efficiency and assignment test employed after a single removal event. For all tests $\alpha = 0.1$. RC 1 = only RW returned to population; RC 2 = RW and {RW, $^{3}/_{4}$RW} returned; RC 3 = RW, {RW, $^{3}/_{4}$RW} and $^{3}/_{4}$RW returned. For reference note level of initial %RW in gene pool for each population and performance of the perfect test (one which correctly identified every sampled individual)

			Sampling efficiency			
			50%		100%	
Level of introgression	Removal criteria	Test/initial conditions	%RW gene pool	% Pop. decline	%RW gene pool	% Pop. decline
Low	RC 1	Initial	93.75	—	93.75	—
		Perfect	96.53	10.0	100.00	20.0
		CPA	96.37	11.9	99.81	23.8
		LR	96.28	10.8	99.51	21.6
		Mixed	96.34	9.1	99.50	18.2
	RC 2	Perfect	96.26	7.5	99.26	15.0
		CPA	96.37	11.9	99.80	23.8
		LR	96.24	8.5	99.24	17.0
		Mixed	96.25	8.1	99.24	16.2
	RC 3	Perfect	95.72	5.0	97.92	10.0
		CPA	95.98	6.7	98.56	13.5
		LR	95.64	5.3	97.75	10.5
		Mixed	95.65	5.3	97.76	10.6
High	RC 1	Initial	81.25	—	81.25	—
		Perfect	86.61	30.0	100.00	60.0
		CPA	86.36	29.7	98.94	59.4
		LR	86.31	27.3	97.48	54.7
		Mixed	86.45	26.2	97.40	52.5
	RC 2	Perfect	86.69	22.5	96.59	45.0
		CPA	86.36	29.7	98.94	59.4
		LR	86.33	24.5	96.29	49.0
		Mixed	86.37	24.2	96.29	48.5
	RC 3	Perfect	85.66	15.0	91.96	30.0
		CPA	85.91	20.2	93.71	40.4
		LR	85.29	15.8	91.19	31.5
		Mixed	85.30	15.8	91.22	31.7

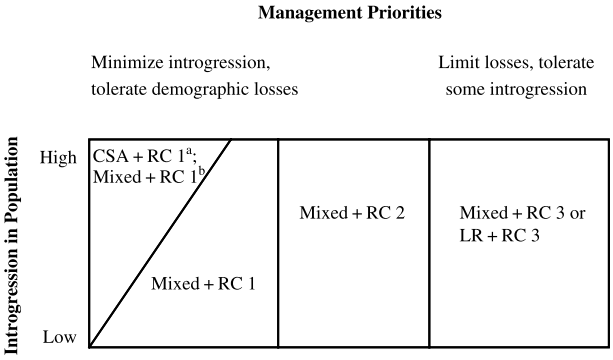

Fig. 2 Best test and removal criteria (RC) as a function of level of introgression in population and management priorities. RC 1 = only RW returned to population; RC 2 = RW and {RW, $^{3}/_{4}$RW} returned; RC 3 = RW, {RW, $^{3}/_{4}$RW} and $^{3}/_{4}$RW returned. Superscripts: [a]best when sampling intensity is high, [b]best when sampling intensity is low.

this context, the more a test is pushed to make definitive assignments (large α) the more often it definitively assigns the wrong identity. Conversely, the more conservatively a test is set (small α), the more often it will make ambiguous calls. For example, compare the assignment patterns of simulated $^{3}/_{4}$RWs under the mixed test with $\alpha = 0.1$ vs. 0.05 (Table 3a vs. 3b). With $\alpha = 0.1$, 9% are misassigned as RWs compared to 4% when $\alpha = 0.05$, the 5% difference having been shifted to {RW, $^{3}/_{4}$RW}. On the other hand, the definitive $\alpha = 0.1$ test correctly assigns 99% of the RWs as RWs, while this number drops to 87% with the more conservative $\alpha = 0.05$ test. The second reason for the tradeoff stems from different assignment patterns between the tests themselves. The mixed test assigns RWs as RWs at the highest rate (99% when $\alpha = 0.1$, Table 2) but it also assigns slightly introgressed canids as RWs at the highest rate (e.g. $^{7}/_{8}$RWs are assigned RW 42% of the time). By contrast, the

CPA test severely limits the rate it assigns introgressed animals as RWs (e.g. only 17% of $^{7}/_{8}$RWs are assigned RW) but the tradeoff is a lower rate of assigning RWs as RWs (94%). The LR test is generally intermediate.

These tradeoffs make it impossible to select a best test based on assignment rates alone. To explore which test is most appropriate for management, it is necessary to consider the impacts of applying them to a population. If the highest priority of the recovery programme is to minimize introgression, removal criteria 1 would be employed. Irrespective of the level of introgression, the CPA test yields the least introgressed postremoval population when sampling intensity is 100% (Table 4). The CPA test also results in the most severe population declines. When introgression is high, the decline reflects positively on the test because nearly all the removals are nonred wolves. For example, of the 59.4% expected decline (Table 4), only 4% are red wolves (details not shown). However, when introgression is low in the population, a substantial proportion of the removals are red wolves (e.g. over 20% of the 23.8% decline are red wolves). When the level of introgression is low, the mixed test yields much lower levels of decline (18%) while producing a gene pool that is nearly as much red wolf as the CPA test (0.3% lower). Currently, the wild gene pool has been estimated at approximately 95% red wolf ancestry, a value near the low level of preremoval introgression considered in the model (93.75%). When the sampling intensity is reduced to 50%, the mixed test becomes the superior test even for moderate to highly introgressed populations. It limits introgression virtually as well as the CPA test and requires a smaller population reduction (Table 4). Another important merit of the mixed test compared to the CPA test is that it is less variable between runs in assigning RWs as RWs (Table 2).

As Table 4 demonstrates, minimizing introgression by removing every animal that is not definitively assigned RW (RC 1) will result in substantial demographic losses. Alternatively, managers might consider allowing a slightly higher degree of introgression in order to reduce losses. For example, a moderate proportion of $^{7}/_{8}$ or even $^{3}/_{4}$RWs might be tolerable. Under RC 3 demographic losses are minimized, but the level of postremoval introgression is also highest. The LR and mixed tests yield virtually identical results. Across levels of introgression and sampling intensity, these tests precipitate the smallest population decline. Although they result in the highest level of postremoval introgression, the introgression level they produce is always very close to the perfect test, indicating they are achieving the balance we are seeking with this removal criterion. RC 2 is intermediate between RC 1 and RC 3 both in terms of population reduction and introgression. Under this removal strategy, the mixed test is always closest to the perfect test irrespective of the level of introgression or sampling intensity. The best test as a function of the level of introgression, management priority and sampling intensity is summarized in Fig. 2.

It must be stressed, however, that the relative performance of the various tests should be viewed as approximations. For several reasons, the numbers in Table 2 should not be interpreted as precise predictions. First, other population compositions can be postulated which produce slightly different numbers and slightly different relative test performances. Second, while the process of introgression is depicted as deterministic, chance plays a role in who is sampled and how they are assigned. Third, the assignment rates (Table 2) come from a simulation and not from the real world. The high accuracy at assigning captive red wolves (Table 1) is a positive sign, but the sample size is small and only a single true identity is represented. The simulation was designed to produce a wide range and large number of hybrid intergrades for estimating rates, not for modelling the population as realistically as possible. Additionally, the number of observations for some identities was very small (e.g. < 5 per run) and estimates are surrounded by large uncertainty (Table 2). We conclude that the assignment rates are likely to be good, but not perfect approximations.

Finally, the process of introgression is dynamic, depending on the pattern of breeding in the population over time. Unless the number of first-generation hybrids (i.e. those involving coyotes) is small and sampling intensity high, then the constant influx of coyote genes will make efforts to limit introgression an uphill battle. One of the most effective ways to limit introgression is to catch and remove the first-generation hybrids. This is also important from a demographic perspective, because the more introgressed coyote genes become in the population, the more accumulated red wolf reproductive effort must be wasted in order to remove them. Because first-generation hybrids are $\leq$ $^{1}/_{2}$RW, the tests should be highly effective at filtering them from the population. Sampling first-generation hybrids should therefore assume a high priority. We are currently exploring the feasibility of using faecal DNA analysis as a way to sample a greater proportion of the population.

Another way to reduce the influx of coyote genes into the red wolf population is to discourage the breeding of coyotes with red wolves (or individuals who are mainly red wolf). Ironically, one factor that may contribute to red wolf–coyote breeding events is the removal of individuals. When removal breaks a breeding pair apart, the remaining red wolf will seek a new mate. Since canids are territorial, the removal of an individual may create an opportunity for a dispersing animal. When this occurs near the perimeter of the population where coyotes are more numerous, the chances are reasonable that the new mate will be coyote. If the removed canid was not highly introgressed (for instance $^{3}/_{4}$RW), it might have been preferable to allow this pair to breed. Alternatively, sterilization can and has been

used in the red wolf recovery programme as a way to stop introgression while not creating an opportunity for a dispersing coyote.

The current strategy of the red wolf recovery programme broadly reflects these genetic, demographic and behavioural considerations. Unidentified canids in the experimental population area are sampled genetically and analysed using the described assignment tests. Those identified as coyotes and first-generation hybrids are removed from the gene pool. Increasing reproductive success among red wolves in recent years has allowed a more aggressive strategy to be taken where animals with smaller degrees of coyote ancestry (e.g. $^{3}/_{4}$RWs) are removed. In the population interior such individuals are generally culled, while along the perimeter they are more commonly sterilized and released. In the long term, managers hope that red wolves will exclude coyotes more effectively as the number, size and stability of red wolf packs grow. This could create a positive feedback where the rate of first-generation hybridization declines steadily.

Assignment tests

Davies *et al*. (1999) concluded that a serious limitation of existing assignment tests is their inability to account for genetic drift when it has occurred between the populations providing allele frequencies and the populations producing the genotypes under analysis. Our approach accounts for genetic drift during this interval. This is accomplished not through qualitative changes in how allele frequencies are estimated or likelihoods calculated, but rather by incorporating drift and mutation into the generation of sampling distributions. In the case of the red wolf, the known pedigree made it possible to improve estimation of the allele frequencies and reduce the uncertainty in how drift has affected the genetic composition of individuals. There are many circumstances where captive breeding, detailed field research and/or extensive parentage analysis produces enough pedigree information to be useful. One intriguing possibility is an assignment test that evaluates the fit between genotype and population (or ancestry) by the ability to place the genotype into the pedigree rather than by calculating a likelihood under Hardy–Weinberg assumptions.

When the pedigree is not known, drift may be accommodated by employing a Wright–Fisher model with a conservatively small effective size before simulating genotypes for estimating sampling distributions. These sampling distributions should be more dispersed than those which assume no drift, reflecting the increasing uncertainty in allele frequencies. This is expected to become important as reference databases age and become several generations or more ancestral to the samples being assigned. Such databases are playing an increasingly important role in wildlife forensics (e.g. Primmer *et al*. 2000; Manel *et al*. 2002), wildlife management (e.g. Nielsen *et al*. 1997; Blanchong *et al*. 2002) and domestic animal science (e.g. Blott *et al*. 1999; Rosenberg *et al*. 2001). The net value of incorporating pedigree information, drift and sampling error in this study is illustrated by the improved assignment rates observed when they are incorporated compared to when they are ignored (Table 3a vs. 3d). We do not suggest that using ancestral reference populations is preferable. When contemporary samples are available, their use with the appropriate test will generally yield greater assignment power.

Several other aspects of our approach warrant mention. First, although observed allele frequencies have been used here (in coyotes), the Bayesian estimator of allele frequencies (Rannala & Mountain 1997) could also be applied easily. Similarly, when population substructure is unclear a priori, boundaries between and allele frequencies within ancestral reference populations could be estimated first using STRUCTURE (Pritchard *et al*. 2000) or the iterative method (Vázquez-Domínguez *et al*. 2001). Drift and mutation would then be simulated before generating the sampling distributions. Second, allele frequency differences between coyotes and the founder red wolves are moderate ($F_{ST} \approx 0.13$). In cases where the reference populations are more differentiated, greater assignment accuracy would be expected or the same accuracy could be achieved with fewer loci. For less differentiated populations, distinguishing closely related intergrades accurately (e.g. $^{3}/_{4}$RW from $^{1}/_{2}$RW) will require more than 20 loci. Finally, the number of private alleles in a genotype is proposed as a method for assigning hybrids (our CPA test). We suggest the advantage of this approach is that, like the distance method of Cornuet *et al*. (1999), it may be less sensitive to the HW assumptions within populations. The disadvantage is that it will usually have lower resolving power than a likelihood-based test (Table 2) when HW is approximated. In this case it was useful to consider CPAs only because red wolves carry virtually no unique alleles. However, the number of private alleles from two populations could be calculated and their joint distribution used to assign ancestry. While the red wolf founder genotypes allows precise definition of private alleles, the possibility that private alleles are an artefact of sampling will typically need to be considered. In simulating the sampling distribution it will be necessary to include alleles absent from the sample — for example, by using Bayesian estimated allele frequencies (Rannala & Mountain 1997) to initiate simulations.

Conclusion

An assignment test approach has been developed which accounts for genetic drift and mutation and makes use of

available pedigree information. Development has occurred in a context where hybridization is the primary focus. However, the test can be made easily to consider multiple populations with or without the possibility of introgression between them. The potential power of the approach is evident in its ability to generally assign both captive red wolves and simulated canids correct identities with a relatively high degree of accuracy. When the potential impacts of applying these rates to an introgressed population are considered (Table 4) several important points emerge. First, the higher the level of introgression, the more difficult and more demographically costly it is to remove introgression from the population. Second, assuming these demographic costs are tolerable, the described tests do have the power to contain introgression — especially when sampling effort is high. Third, the projected assignment rates a decade in the future are very similar to the current assignment rates suggesting that the effectiveness of the tests should not decay substantially for at least several generations (Table 3c). Finally, the choice of optimal test and removal criteria depends on how limiting introgression is prioritized compared to limiting demographic losses, and to a lesser extent on the current level of introgression in the population. The issue of weighing demography against introgression is a complex one which we do not address here. Nevertheless, the most effective way to limit both introgression and demographic losses is to discourage first-generation hybridization events and catch them as, or soon after, they occur. The tests are, without exception, highly effective at identifying first-generation hybrids.

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Appendix I

The algorithm used to generate genotypes of known ancestry proceeds year by year according to the following steps.
(1) *Define breeding pool*. Potential breeders are defined as all living individuals born at least 2 years previously. Males and females in the pedigree for which death date is known are included in the breeding pool if they survived past 1 February and 15 May, respectively. If death data are unknown for a pedigreed individual, a longevity value is assigned at the beginning of the simulation by drawing a value from Table A1. This distribution is a compilation from all pedigreed animals born in the wild prior to 1995 for which a death date is known. If no death date exists for an individual but it is known to have survived at least x years based on field observations, then its longevity is drawn from the conditional distribution given x.
(2) *Pair formation*. Pairs are formed in four steps.

(a) Pairs known to have produced pups that year based on the pedigree are established.
(b) Pairs from the previous year not joined in step (a) are re-paired with probability 0.75.
(c) A predetermined number of RW × Coy pairs for that year are established (Table A2). This ensures an influx of coyote genes. Red wolves are random draws from pedigree individuals who produced pups, but with an unknown partner. If an insufficient number exist in the pedigree, then red wolves are drawn at random from unpaired red wolves in the breeding pool. Opposite sex coyotes for these pairings are generated by drawing alleles from a bootstrap resampled distribution of coyote allele frequencies. Unless coyotes re-pair [step (b)] they are assumed to survive only through this year. Although somewhat unrealistic, this creates greater independence between observations.
(d) A predetermined number of 'other' pairings (Table A2) are simulated. This step generates the various intergrades of introgression (e.g. $^{7}/_{8}$, $^{3}/_{4}$, $^{5}/_{8}$, $^{3}/_{8}$, $^{1}/_{4}$RW) as well as allowing the nonpedigreed population to expand and interbreed with individuals in the pedigree. Pedigreed individuals who produced pups with an unknown partner [assuming any remain after step (c)] are drawn at random and paired with a random unpaired individual of the opposite sex from the breeding pool (without regard to ancestry). If this does not create enough 'other' pairs, then pairs are formed by randomly drawing an individual of each sex from the breeding pool.

(3) *Assign litter size*. All pairs are assigned a litter size. For pairs derived from the pedigree, the litter size and the identity of the pups is defined by the pedigree itself. Otherwise a litter size is drawn at random from the distribution in Table A3. This distribution is derived empirically from all known litters born in the wild between 1987 and 2001.
(4) *Assign genotype*. Each offspring receives a genotype by drawing a random allele from each parent at each locus. The previously described mutational model is employed for each allele at each locus.
(5) *Assign longevity*. Each offspring of each litter is assigned an independent longevity (Table A1).

It is important to note that the values in Table A2 probably do not accurately describe breeding patterns in the wild. Instead, they are used because they produce enough litters of various intergrades in the simulations for assignment rate estimation while keeping the number of pairs and the total population size reasonable (Fig. A1).

Because no pedigree exists for the future, the simulation must be modified to simulate the population after 2001. After 2001 pair formation occurs under a different set of steps.

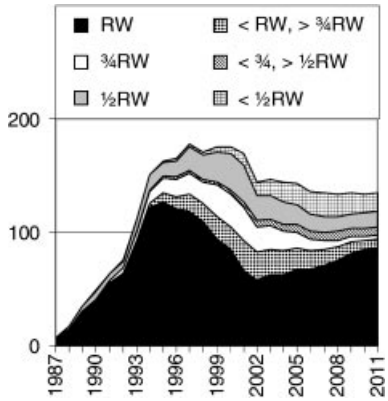

Fig. A1 Mean size of simulated population (excluding coyotes) 1987–2011 broken down by level of introgression.

Table A1 Cumulative distribution of longevity used in simulations

Lifespan	Cumulative probability
0	0.265
1	0.382
2	0.441
3	0.529
4	0.662
5	0.721
6	0.809
7	0.882
8	0.985
9	0.985
10	0.985
11	1.000

Table A2 Number of simulated RW × Coy pairings and 'Other' pairings by year. A larger number of other pairings were used in the early 1990s to build the number of breeding pairs up to more than 10, thereby ensuring an adequate number of simulated individuals of various degrees of introgression for analysis

Year	No. of RW × Coy pairings	No. of other pairings
1987	0	0
1988	1	2
1989	1	2
1990	1	2
1991	2	3
1992	2	3
1993	2	3
1994	2	5
1995	2	5
1996	3	1
1997	3	1
1998	3	1
1999	3	1
2000	3	1
2001	3	1
2002	2	1
2003	2	1
2004	2	1
2005	2	1
2006	2	1
2007	2	1
2008	2	1
2009	2	1
2010	2	1
2011	2	1

Table A3 Cumulative distribution of litter size used in simulations

Litter size	Cumulative probability
1	0.230
2	0.443
3	0.639
4	0.787
5	0.902
6	0.934
7	0.984
8	1.000

(a) Pairs from the previous year repair with probability 0.75.
(b) A predetermined number of red wolves (Table A1) are simulated by pairing random red wolves of each sex from the breeding pool. Pairs created in step (a) do not count toward this total. If an insufficient number of red wolves of either sex are in the breeding pool then the number of red wolf pairs is fewer than the predetermined number.
(c) The predetermined number of RW × Coy pairs (Table A2) are created by drawing a random red wolf from the breeding pool and simulating a coyote (see above).
(d) The predetermined number of other pairs (Table A2) are created by drawing a random individual of each sex from the breeding pool without regard to ancestry.

All other steps in the algorithm remain the same.