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Assessing the prevalence of hybridization between sympatric *Canis* species surrounding the red wolf (*Canis rufus*) recovery area in North Carolina

JUSTIN H. BOHLING and LISETTE P. WAITS

Department of Fish and Wildlife Resources, University of Idaho, Moscow, ID 83844-1136, USA

Abstract

Predicting spatial patterns of hybridization is important for evolutionary and conservation biology yet are hampered by poor understanding of how hybridizing species can interact. This is especially pertinent in contact zones where hybridizing populations are sympatric. In this study, we examined the extent of red wolf (*Canis rufus*) colonization and introgression where the species contacts a coyote (*C. latrans*) population in North Carolina, USA. We surveyed 22 000 km^2 in the winter of 2008 for scat and identified individual canids through genetic analysis. Of 614 collected scats, 250 were assigned to canids by mitochondrial DNA (mtDNA) sequencing. Canid samples were genotyped at 6–17 microsatellite loci (nDNA) and assigned to species using three admixture criteria implemented in two Bayesian clustering programs. We genotyped 82 individuals but none were identified as red wolves. Two individuals had red wolf mtDNA but no significant red wolf nDNA ancestry. One individual possessed significant red wolf nDNA ancestry (approximately 30%) using all criteria, although seven other individuals showed evidence of red wolf ancestry (11–21%) using the relaxed criterion. Overall, seven individuals were classified as hybrids using the conservative criteria and 37 using the relaxed criterion. We found evidence of dog (*C. familiaris*) and gray wolf (*C. lupus*) introgression into the coyote population. We compared the performance of different methods and criteria by analyzing known red wolves and hybrids. These results suggest that red wolf colonization and introgression in North Carolina is minimal and provide insights into the utility of Bayesian clustering methods to detect hybridization.

Keywords: Bayesian clustering, endangered species, genetic introgression, hybrid zones, non-invasive genetic sampling

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Introduction

Hybridization is a powerful evolutionary force that can shape local adaption and encourage speciation (Moore 1977; Barton & Hewitt 1985, 1989; Arnold 1997; Dowling & Secor 1997). From a conservation perspective, however, it can erode species boundaries and lead to the homogenization of distinct populations (Rhymer & Simberloff 1996; Allendorf *et al.* 2001). Numerous rare and endangered species are threatened by interspecific hybridization and genetic introgression (Rhymer & Simberloff 1996; Allendorf *et al.* 2001; Randi 2008; Seehausen *et al.* 2008). Despite increasing knowledge about hybridization and its recognition as a conservation issue, formulating comprehensive management strategies to limit hybridization has proven to be a challenge (Allendorf *et al.* 2001).

A major impediment to mitigating the effects of hybridization is that the interactions between hybridizing species are often poorly understood. Anthropogenic processes, such as the transport of non-native species and the breakdown of reproductive and/or ecological barriers, are known to facilitate hybridization (Rhymer & Simberloff 1996; Allendorf *et al.* 2001; Seehausen *et al.* 2008), but the actual process is dependent on how spe-

Correspondence: Justin H. Bohling, Fax: (+1) 208 885 9080; E-mail: jhbohling@vandals.uidaho.edu

cies interact when they come into contact. Seehausen *et al.* (2008) reviewed numerous examples where changes in ecological conditions due to human activity led to unpredicted hybridization between species. A major challenge is predicting whether contact between potentially hybridizing species will result in reproductive isolation (Turelli *et al.* 2001; Johnson 2006), the formation of a stable hybrid zone (Barton & Hewitt 1989; Howard & Waring 1991; Arnold 1992) or a hybrid swarm that leads to the fusion of the two species (Seehausen 2004). Each of these scenarios has different implications for conservation biologists.

Another challenge in understanding hybridization is our ability to identity hybrids using different techniques. Historically, hybrids were identified using morphometric characters, but advances in genetics and molecular biology have provided new methods to evaluate hybrid ancestry using mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) markers. The use of hypervariable nDNA molecular markers and the development of new statistical techniques, especially Bayesian assignment tests, provide new opportunities to discern fine-scale patterns of genetic ancestry at the individual level (Pearse & Crandall 2004; Manel *et al.* 2005). Despite these advances, challenges still exist in detecting hybridization. Simulation studies have shown that the accuracy of Bayesian clustering methods in assigning individuals to populations and detecting introgression is dependent on a variety of factors, including the number of loci, degree of differentiation between populations, and number of hybrid generations (Latch *et al.* 2006; Vaha & Primmer 2006; Sanz *et al.* 2009). Furthermore, classification of hybrids using these methods is dependent on thresholds of ancestry values that are often subjectively predetermined by researchers (Vaha & Primmer 2006).

Challenges associated with understanding the interactions between hybridizing species and identifying hybrids have played a major role in the recovery of the red wolf (*Canis rufus*). The red wolf is a critically endangered species that formerly existed across the eastern United States (Paradiso & Nowak 1972; USFWS 1990; Nowak 2002; Kyle *et al.* 2006) but was driven to near extinction by overharvest, habitat loss, and hybridization with expanding coyote populations (*C. latrans*) (McCarley 1962; Paradiso & Nowak 1972; USFWS 1990). After being declared extinct in the wild and placed into an intensive captive breeding program, the red wolf was reintroduced by the US Fish and Wildlife Service (USFWS) into northeastern North Carolina beginning in 1987 to reestablish a wild population (Phillips & Parker 1988; USFWS 1990, 2007; Phillips *et al.* 2003; Stoskopf *et al.* 2005). Currently about 100–120 red wolves occupy the experimental population area within the 688 000 hectare Albemarle Peninsula in northeastern North Carolina (USFWS 2007).

Prior to the red wolf reintroduction program, coyotes expanded their range across the eastern United States (Hill *et al.* 1987; Phillips *et al.* 2003), and in 1993 the first hybridization event between reintroduced red wolves and a coyote was detected (Kelly *et al.* 1999; Adams 2006). Population modelling (Kelly *et al.* 1999; Fredrickson & Hedrick 2006) and observations from the red wolf's historic decline (McCarley 1962; Paradiso & Nowak 1972; Nowak 2002, 2003) suggested that a small red wolf population, without management of coyotes, would become enveloped in a hybrid swarm. An aggressive adaptive management plan to minimize hybridization and introgression was implemented beginning in 1999 and has been successful in limiting coyote introgression into the red wolf gene pool (Beck 2005; Stoskopf *et al.* 2005; Adams 2006; USFWS 2007).

The USFWS only has authority to manage red wolves within the current range of the Non-Essential Experimental Population (NEP), which covers Dare, Hyde, Tyrrell, Washington, and Beaufort counties in North Carolina (Fig. 1a). However, there is no physical boundary to prevent red wolf movement from the NEP into the remainder of North Carolina. Red wolves have been documented dispersing beyond the official range of the NEP throughout the 20-year recovery period (Phillips *et al.* 2003), up to160 km from their birth site (A. Beyer, personal communication). Over the past decade, the red wolf population has produced 25–50 known pups annually; however, many of these pups are never recaptured as adults (USFWS 2007). Population trends since 2000 suggest that the red wolf population has plateaued within the NEP, perhaps due to saturation of quality red wolf habitat (USFWS 2007). Given the dispersal capabilities of the species and high pup production over the last decade, we hypothesized that wolves have moved beyond the boundary of NEP and expanded into adjacent territories providing the opportunity for hybridization and introgression of red wolf genes into the local coyote population.

Our objectives in this study were to (i) determine if red wolves had colonized the region outside the NEP; (ii) assess the prevalence and spatial distribution of hybridization between red wolves and coyotes in this region; and (iii) compare different molecular and statistical methods for detecting hybridization and introgression. Our study evaluated the important management question of whether red wolves have the ability to colonize an area saturated with coyotes and remain reproductively isolated. More broadly, our study provides insight into the patterns of hybridization and introgression between two expanding canid populations and

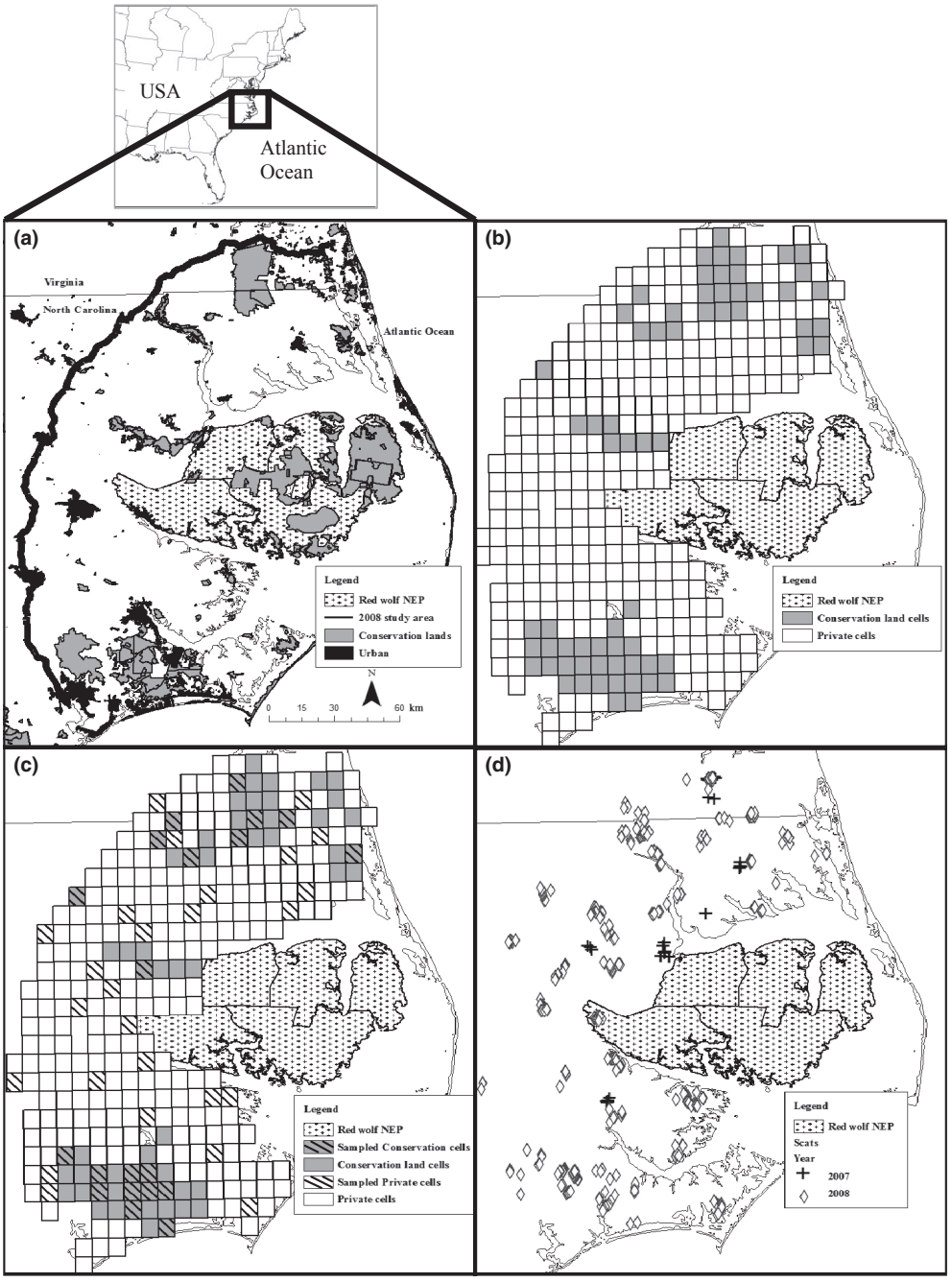

Fig. 1 (a–d) Map of eastern North Carolina and the implemented study design.

explores the limitations and challenges of detecting hybridization and introgression using genetic techniques.

Methods

Study area

Our study area included 22 counties in northeastern North Carolina and five counties in southeastern Virginia (Fig. 1a) that are within recorded red wolf dispersal distances (Phillips *et al.* 2003). The cities of Chesapeake, VA and Jacksonville, NC served as the northern and southern boundaries of the study area, respectively. Highway 258 (Study Area Boundary, Fig. 1a), which runs through both NC and VA, constituted the western boundary of the study area, which is near the western limit of known red wolf dispersal events over the past 20 years (A. Beyer, personal communication). The five counties that compose the offi-

cial range of the red wolf NEP were not included in the study area.

The study area is ecologically similar to the NEP area and is characterized as coastal lowlands dominated by bottomland swamps and agricultural areas, most of which are privately owned (Fraver 1994; Kelly *et al.* 2004). However, there are several large tracts of undeveloped land owned by public entities and the portion of the study area in North Carolina contains one of the lowest human population densities in the state (US Census Bureau 2001).

Study design

To provide an unbiased and efficient assessment of the canid population, non-invasive genetic sampling (NGS) of fecal material (scat) was utilized to identify individual canids. NGS has been used with high success within the red wolf NEP to locate coyotes and red wolf-coyote hybrids (Adams *et al.* 2003a, 2007; Miller *et al.* 2003). To evenly distribute sampling, the study area was divided into 326 grid cells 5 min longitude by 5 min latitude with the Hawth's Tools Extension (Beyer 2004) for ArcGIS (ESRI) (Fig. 1b). These cells were stratified based upon the presence of conservation areas (e.g. National Forests, wildlife refuges, state game lands, etc.). This stratification was designed to balance land access while ensuring even sampling across the study area because we could not obtain access to all private lands. Any grid cell with 10% or more of its area covered by a conservation area greater than 10 km^2 in size (minimum home range of coyotes in Georgia, Holzman *et al.* 1992) was classified as a 'Conservation Land cell'. Thus, two types of cells were identified across the study area: cells with conservation lands ('Conservation land cells') and cells without conservation lands ('Private cells'; Fig. 1b). Because this region is bordered by the ocean and contains pockets of urban development, several cells were predominantly composed of open water or cities that were unsuitable for sampling or provided no means of access. Grid cells were removed if >40% of their area was composed of urban areas and/or they contained <10 km^2 of land due to coastlines. From the remaining grid cells, 30% of the 'Conservation Land' cells and 10% of the 'Private cells' were randomly selected to compose the official survey (Fig. 1c), which provided an approximately equal number of 'Conservation Land' and 'Private cells'.

Non-paved roads within each grid cell were identified using maps and U.S. Census Bureau data (US Census Bureau 2000). Canids within the NEP use rural non-paved roads as travel corridors and territory boundaries and frequently deposit scats on these roads (Adams *et al.* 2003a, 2007). Proportional-probability sampling (Williams *et al.* 2002) was used to randomly select 10 km of roads in each grid cell to standardize sampling intensity across all cells. In grid cells with less than 10 km of roads, all non-paved roads were sampled. There were several instances when land owners did not permit access to roads that had been selected; in those situations, new roads were randomly selected to fill the 10 km threshold.

Scat was collected from mid-January to mid-March in 2008, which corresponds to the breeding season for red wolves (Phillips *et al.* 2003). A small piece of fecal material ($\sim$1 cm^2) from the outside surface of the scat was removed from each scat using metal tweezers and then placed in a 2.0 mL screw-top tube containing 1.2 mL DET buffer (Frantzen *et al.* 1998; Stenglein *et al.* 2010). Tweezers were exposed to an open flame before and after use to prevent cross-contamination between samples.

Species identification

We closely followed laboratory methods previously developed for scat surveys within the NEP, as outlined in Adams *et al.* (2003a, 2007), Miller *et al.* (2003), and Adams & Waits (2007). DNA was extracted from scats using the QiAmp Stool Kit (Qiagen, Valencia, CA, USA) in a laboratory dedicated to low-quality DNA samples. Negative controls were included to monitor for contamination. Species identification was conducted by sequencing a 200 bp fragment of the mtDNA control region using the primers ScatSeqF and ScatSeqR (Adams *et al.* 2003a). This fragment was also useful for detecting signals of past hybridization because all female founders of the red wolf captive breeding program possessed a single unique haplotype for this fragment that to date has not been observed in any other canid (Adams *et al.* 2003a). The species of origin was identified by querying each sequence in the NCBI MegaBLAST database. Matches were accepted when >85% of the bases matched a taxon.

Microsatellite genotyping

When scat samples tested positive for *Canis* mtDNA, individual identification was conducted by screening samples at nine microsatellite loci (CXX172, CXX173, CXX20, CXX200, CXX109, CXX250, Ostrander *et al.* 1993; AHT103, AHT121, Holmes *et al.* 1995; CXX377, Mellersh *et al.* 1997) in a single multiplex reaction similar to Adams *et al.* (2007) using the Qiagen multiplex kit. To minimize genotyping errors, we utilized an approach similar to the reference genotype method described by Adams & Waits (2007). Initially two PCRs were performed using multiplex 1 and scats that failed to

amplify at five or more loci were removed from further analysis. If the genotypes differed between the two PCRs, up to five more amplifications were performed as needed. Heterozygous genotypes were accepted if each allele was observed in two independent PCRs; homozygous genotypes were accepted if the genotype was observed in three independent PCRs. Success rates and error rates for each locus were calculated following Broquet & Petit (2004). For the first multiplex, the maximum observed false allele rate for any locus was 5% and the allelic dropout rate per locus ranged from 14% to 22% except for locus CXX109, which had an allelic dropout rate of 42%. Due to the high likelihood of observing false homozygotes at this locus, we only accepted a homozygous genotype if it was observed in four independent PCRs ($P = 0.03$ of observing four false homozygotes). Also, we did not accept a consensus homogenous genotype at CXX109 if a heterozygous genotype was observed in one PCR run. We did run our scat genotypes through STRUCTURE (see below) without this locus and it had little impact on ancestry estimates: the average absolute difference in q values between the two runs was 0.01 ± 0.017 SD. Therefore, we decided to include this locus in our final genotyping.

Because there was a possibility of multiple recaptures per individual, the probability of identity for siblings (PID_{Sibs}, Waits *et al.* 2001) was calculated using Gimlet 1.3.3 (Valiere 2002) to determine the minimum number of loci needed to differentiate individual genotypes of closely related individuals. With the four known canid groups (see below) serving as the reference populations, the estimated PID_{Sibs} at six loci was sufficiently low (0.003–0.006) to differentiate individuals. Duplicate genotypes were regrouped using Gimlet to identify unique individuals.

After genotyping the initial multiplex, ancestry (q) was estimated for each unique genotype using STRUCTURE 2.2 (Pritchard *et al.* 2000; Falush *et al.* 2003) following the methods described below. Fifty-one individuals with evidence of mixed ancestry ($q < 0.90$) were amplified at a second microsatellite multiplex containing eight additional loci (CXX2054, CXX2062,CXX2001, CXX2004, CXX2010, CXX2145, Mellersh *et al.* 1997; CXX225, Ostrander *et al.* 1993; CXX403, Ostrander *et al.* 1995) to provide additional resolution. Allelic dropout rate per locus for the second multiplex ranged from 0% to 27% and false allele rate ranged from 0% to 4%.

Evaluating hybridization

Genetic ancestry of individuals was assessed using the Bayesian clustering programs STRUCTURE 2.2 (Pritchard *et al.* 2000) and BAPS 5.1 (Corander *et al.* 2003, 2006) using representatives of each species as training sets (*sensu* Hauser *et al.* 2006). Several simulation studies have found that these clustering programs can provide incongruent results for the same dataset and advise using multiple programs when evaluating admixture (Vaha & Primmer 2006; Sanz *et al.* 2009). The training sets consisted of 94 coyotes from North Carolina, Virginia, and Texas (Miller *et al.* 2003); 38 grey wolves (*Canis lupus*) from Idaho and Alaska; 28 domestic dogs; and 154 red wolves that were identified as pure descendents of the red wolf founders through pedigree analysis (Adams 2006). To ensure that individuals in our training sets were 'pure' members of their respective groups, we initially analysed our putative known groups using STRUCTURE and removed individuals with less than 95% posterior assignment to their putative species. In this analysis, each individual was assigned to its putative species with the designation POPFLAG = 1 (Pritchard *et al.* 2003) and the 'Use Population Information' option was used as the ancestry model with the default parameter settings. The number of populations was set to four with a burn-in period of 100 000 reps followed by 1 000 000 MCMC reps. Five replicates of this model were conducted to evaluate variation in ancestry estimates (q) but we found almost no variation between replicates (Standard Deviation = 4.13×10^{-5}). Thus, we report ancestry results from one iteration of STRUCTURE. After running the training sets, 82 coyotes, 37 grey wolves, 27 dogs, and 151 red wolves were classified as 'pure' based on this 95% threshold. These known individuals then served as the training sets for both STRUCTURE and BAPS when ancestry was estimated for the unknown scat genotypes.

Ancestry for all unique individuals genotyped from scat samples was estimated in STRUCTURE using the same process and parameter sets outlined above, except scat genotypes were assigned a POPFLAG = 0, indicating unknown ancestry. Only the individuals of known ancestry were assigned a POPFLAG designation of 1, meaning only those individuals were used to estimate allele frequencies. Ancestry was then estimated for each potential species group in the form of a q value. Ancestry within a species group was considered significant with STRUCTURE using two different criteria – conservative and relaxed. The more conservative criterion considered ancestry significant if the 90% credibility interval surrounding a q value did not overlap with 0; thus, individuals were classified as admixed if the credibility intervals for two or more genetic groups did not overlap 0. Credibility intervals surrounding the point estimates were frequently wide and overlapped 0 even if the q value was suggestive of admixture. Thus, we used a second more relaxed criterion in which an individual was considered admixed if it had a q-value of

0.1 or greater for two or more species. Typically, studies evaluating hybridization using Bayesian clustering programs rely solely on setting arbitrary thresholds for q values when determining admixture (Vaha & Primmer 2006). The 0.1 q value threshold was used for this study because it provides a relatively relaxed criteria for classifying hybrids compared to the other methods and this value has been frequently used in the literature (e.g. Beaumont *et al.* 2001; Vaha & Primmer 2006; Barilani *et al.* 2007; Oliveira *et al.* 2008; Trigo *et al.* 2008; Sanz *et al.* 2009; Yokoyama *et al.* 2009). However, the ability to utilize Bayesian credibility intervals (similar to likelihood-based confidence intervals) around these q values provides insight into the statistical support for those ancestry coefficients and a more conservative measure of admixture (Pritchard *et al.* 2000; Beaumont *et al.* 2001; Trigo *et al.* 2008; Yokoyama *et al.* 2009).

For BAPS, the fixed K clustering option was enabled and a 'Trained clustering' analysis was conducted using known individuals from the four predefined species groups as the prior information and unknown scat genotypes as the sampling units (Corander *et al.* 2006). The upper bound on the number of distinct clusters was set to $K = 4$. These results were used to conduct an 'Admixture based on mixture clustering' analysis to estimate ancestry coefficients. The simulations consisted of 100 iterations to estimate ancestry coefficients for our genotypes, 200 simulated reference individuals from each population, and 20 iterations to estimate ancestry coefficients for these reference individuals. BAPS classifies unknown individuals into the known groups unless there is statistically significant ($P < 0.1$) evidence for admixture (Corander & Marttinen 2006; Corander *et al.* 2006); thus, this was the criterion we used to classify an individual as a hybrid using this software program.

Evaluating programs using samples of known ancestry

We tested the accuracy of STRUCTURE and BAPS by running these programs with genotypes of puppies of known ancestry that were born within the NEP during 2008 and 2009. Since this wild red wolf population was founded by captive individuals, active monitoring by the USFWS and molecular techniques have allowed for an extensive reconstruction of the pedigree for this population (Adams 2006). Every spring, blood samples are collected from red wolf puppies and assigned to parents using this pedigree and field data from the USFWS. Ancestry was determined for individuals based upon the ancestry of their parents. Twenty-four individuals from five litters were determined to be 100% pure red wolves because both parents could be traced to the original 14 red wolf founders, and 17 individuals from four litters were classified as ~50% hybrids between red wolves and coyotes based on the pedigree. For all of the hybrids used in this test, the only parents that were identified were female red wolves. Hybrid ancestry was confirmed for these puppies using a maximum-likelihood based test (Miller *et al.* 2003): 11 were classified as F1 hybrids and six as coyote × F1 hybrid crosses. Since the males involved in these hybrid litters were never located, we assumed that the fathers of these litters were 100% pure coyotes. Two of four the female red wolves involved in these hybrid events were not 100% pure red wolves: they are descendants of a hybrid male that backcrossed with the red wolf population (see Stoskopf *et al.* 2005). One hybrid puppy had a 93.75% red wolf mother and six had a 96.85% red wolf mother (Table 2).

We analysed these samples in STRUCTURE and BAPS using the same parameters and training sets as the scat genotypes. Since number of loci can play a large role in the accuracy of these programs (Vaha & Primmer 2006), we tested these puppies at the full suite of 17 loci and also at 6 loci, which was the minimum number of loci we would accept for a scat genotype. To mimic the genotypes we typically found for scats, the six loci we selected for this test were the six that amplified most frequently for scats. All six of these loci were amplified in >90% of the scat genotypes; no other loci had amplification rates >70%.

Results

Scat collection

Twenty-six 'Private cells' and 20 'Conservation Land cells' were randomly selected for the survey and every selected cell was sampled except for two 'Private cells' that had limited road access. The threshold of 10 km of roads was reached in every cell except two and overall 435.07 km of roads were surveyed. The number of scats collected per cell varied from two to 45 with an average of 13, leading to a total of 571 scats collected for the entire survey. We also added 44 scats collected during an October 2007 pilot survey to the dataset, so 614 scats were collected for genetic analysis (Fig. 1d).

MtDNA sequencing and microsatellite genotyping

MtDNA sequences were obtained from 492 scats (80.7%) that were assigned to 13 mammalian species (Table 1, Table S3, Supporting information). Of these 492 scats, 250 (50.8%) were classified as canid and five produced sequences that matched the unique red wolf haplotype (Table 1). Fifty-seven scats were assigned as swamp rabbit (*Sylvilagus palustris*), likely due to amplification of prey DNA. All 57 of these scats were rese-

Table 1 Species identified based on mtDNA sequencing of scats

Species	Number of scats
Canis latrans	220
C. rufus	5
C. lupus	7
C. familiaris	18
Vulpes vulpes	157
Lynx rufus	10
Ursus americanus	3
Felis domesticus	3
Lontra canadensis	3
Procyon lotor	8
Neovison vison	1
Sylvilagus palustris	57
Failed	118
Total	614

quenced using a different primer set (L16345 and H16571, Ward *et al.* 1991) and bobcat (*Lynx rufus*) control region sequences were found in 23 samples. The remaining samples did not produce readable sequences.

Consensus microsatellite genotypes at six or more loci were obtained for 135 of the 250 canid scats (54%). Each unique individual was genotyped at an average of 10.2 loci (range 6–17) and 82 unique individual canids were identified. Approximately 43% of all individual genotypes were only detected once, and the maximum number of recaptures was nine.

Detecting red wolf ancestry

As noted, five scats produced haplotypes that matched the unique red wolf mtDNA haplotype (Table 1). Four out of these five scats were collected on the same road and microsatellite genotyping revealed that they belonged to the same individual; thus, only two individuals out of 82 possessed the red wolf mtDNA haplotype. Both of these individuals were classified as pure coyotes by BAPS and STRUCTURE-conservative (Table 2a and Fig. 2a). However, one individual with red wolf mtDNA (Canid029) was classified as admixed (34.6% red wolf) using STRUCTURE-relaxed (Fig. 2a). This individual was genotyped at only six loci.

No individual was classified as a pure red wolf by any of the three criteria. However, one individual (Canid040) had evidence of statistically significant red wolf ancestry using all three criteria. For Canid040, the estimates of ancestry coefficients from BAPS (coyote $q = 0.66$, red wolf $q = 0.34$) and STRUCTURE (coyote $q = 0.58$, red wolf $q = 0.34$) were similar (Table 2a and

Table 2 BAPS ancestry coefficients and mtDNA halotypes for (a) individuals with evidence of red wolf ancestry (mtDNA or nDNA) using any of the three Bayesian criteria or haplotype analysis, (b) individuals with evidence of multi-species ancestry (mtDNA or nDNA) using BAPS, the STRUCTURE-conservative criterion, or mtDNA haplotype analysis

	BAPS ancestry coefficients					
Individual	*Coyote*	*Grey wolf*	*Dog*	*Red wolf*	Haplotype	Loci amplified
(a)						
Canid019	1	0	0	0	Coyote	13
Canid029	1	0	0	0	Red wolf	6
Canid040	0.66	0	0	0.34	Coyote	15
Canid047	0.32	0.45	0.07	0.16	Coyote	13
Canid053	1	0	0	0	Coyote	15
Canid060	1	0	0	0	Red wolf	13
Canid068	0	0	1	0	Coyote	6
Canid076	1	0	0	0	Coyote	9
Canid080	1	0	0	0	Coyote	13
(b)						
Canid005	0	0	1	0	Dog	6
Canid010	0	0	1	0	Dog	11
Canid016	0	0	1	0	Coyote	7
Canid022	0	0	1	0	Coyote	7
Canid030	0.59	0.31	0.05	0.05	Coyote	11
Canid042	0	0	1	0	Coyote	11
Canid055	0	0	1	0	Dog	14
Canid059	0.46	0	0.53	0.01	Coyote	13
Canid079	0	0	1	0	Coyote	7
Canid081	0	0	1	0	Dog	14

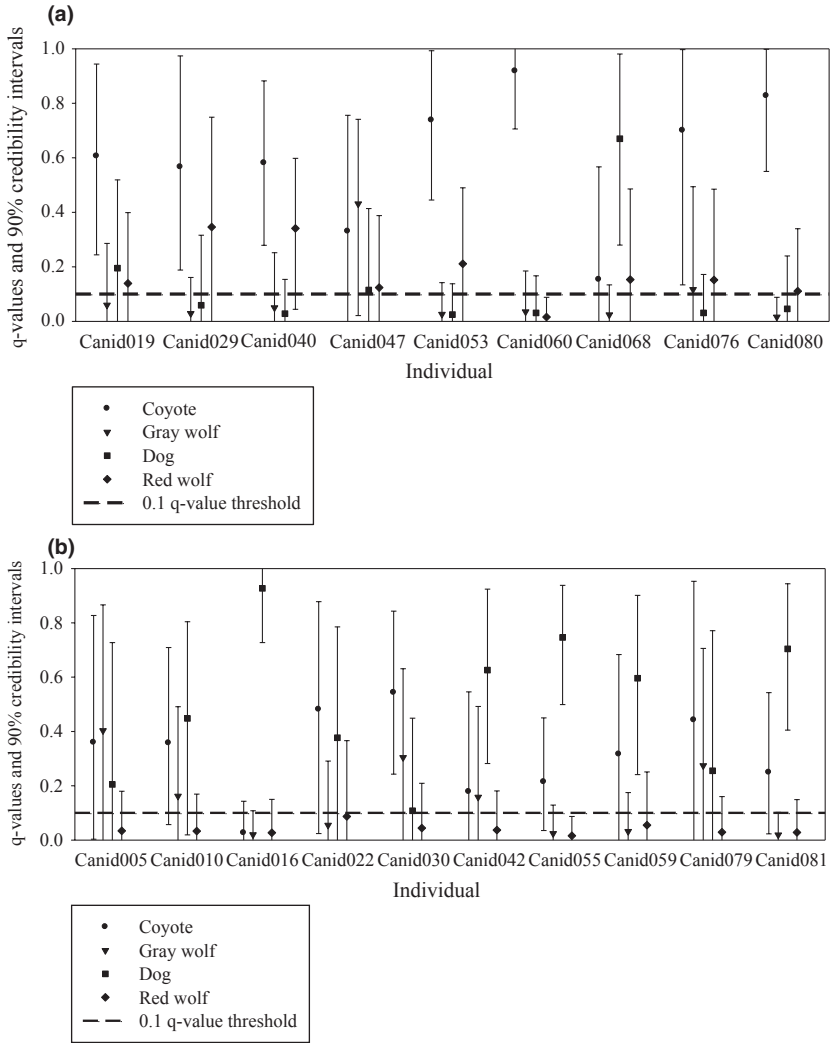

Fig. 2 STRUCTURE ancestry estimate *q* values and 90% credibility intervals estimated from nDNA microsatellite data for (a) individuals with evidence of red wolf ancestry (mtDNA or nDNA) or (b) individuals with evidence of multi-species ancestry using BAPS, STRUCTURE-conservative, or mtDNA sequencing. Point estimates of *q*-values are denoted by the black symbols and the associated credibility intervals by the lines extending from those symbols.

Fig. 2a). This individual possessed a coyote mtDNA haplotype. Six other individuals had evidence of red wolf ancestry using STRUCTURE-relaxed with red wolf *q* values ranging from 0.111 to 0.211 (Fig. 2a). All these individuals had coyote mtDNA haplotypes, and all but one (Canid 047) were classified as coyotes by BAPS.

Table 3 Number of individual canids assigned to each ancestry group using three admixture criteria implemented in the Bayesian software packages BAPS and STRUCTURE. Results are based on data derived from 6–17 nuclear DNA microsatellite loci.

Genetic group	BAPS	STRUCTURE-conservative	STRUCTURE-relaxed
Coyote	63	66	39
Grey wolf	1	1	0
Domestic dog	14	10	6
Red wolf	0	0	0
Admixed	4	4	37
No assignment	0	1	0

Comparing admixture analyses

Estimates of individual ancestry and hybridization varied dramatically depending on the software program and the criterion that were used. Both BAPS and STRUCTURE-conservative classified four of the 82 individuals as admixed, but only one of these was the same individual (Canid040). Under the STRUCTURE-relaxed criterion, the number of individuals considered admixed increased to 37 (Table 3). Every individual classified as admixed by BAPS and the STRUCTURE-conservative criteria was also classified as admixed using STRUCTURE-relaxed. Seventy-two of the 82 individuals, including one admixed individual (Canid040), were assigned to the same species using both BAPS and the STRUCTURE-conservative criterion.

Six individuals were classified as admixed under one criterion but 'pure' under the other. Using BAPS, one

individual was classified as a coyote-dog hybrid (Canid059) (Table 2b), another as a coyote-grey wolf hybrid (Canid030), and the third as a multi-species hybrid (Canid047) (Table 2a). STRUCTURE produced *q* values for these individuals that were similar to ancestry coefficients estimated by BAPS (Fig. 2b); however, these individuals would not have been classified as admixed using the STRUCTURE-conservative criterion. All three individuals had coyote mtDNA haplotypes. The STRUCTURE-conservative criterion classified three different individuals as admixed. Two of these individuals (Canid055 and Canid081) had evidence of mixed coyote and dog ancestry and the third (Canid010) had a mix of coyote, dog, and grey wolf ancestry (Fig. 2b). However, these individuals were classified as dogs by BAPS and had dog mtDNA haplotypes.

In two cases BAPS classified an individual as a 'pure' member of one genetic group but STRUCTURE-conservative suggested it was a 'pure' member of another group (Fig. 2b and Table 2b). Canid005 was classified as a grey wolf by BAPS but as a coyote by STRUCTURE-conservative. STRUCTURE *q* values for Canid005 were indicative of mixed ancestry (Fig. 2b) and it had a dog mtDNA haplotype. The other individual (Canid022) was grouped with the dogs by BAPS but as a coyote by the STRUCTURE-conservative method. According to STRUCTURE-relaxed criterion this individual appeared to be a coyote-dog hybrid (Fig. 2b) with a coyote mtDNA haplotype. Both of these individuals were genotyped at only six and seven loci, respectively. One individual (Canid079) had credibility intervals that overlapped 0 for all four genetic groups (Fig. 2b); however, it was only genotyped at seven loci. Canid079 appeared to be of mixed ancestry according to STRUCTURE-relaxed (Fig. 2b) but was identified as a grey wolf by BAPS.

Comparison of nuclear DNA ancestry and mitochondrial haplotype showed that seven individuals classified as 'pure' by BAPS possessed mtDNA haplotypes assigned to another species. Five of these individuals were 'dogs' with coyote haplotypes and two were 'coyotes' with the red wolf haplotype (Canids 29 and 60). Four out of these five 'dogs' were classified as admixed using STRUCTURE-relaxed, but not using STRUCTURE-conservative (Fig. 2b).None of the animals classified as 'pure' using the STRUCTURE-relaxed criterion showed incongruence between nDNA ancestry and mtDNA haplotype.

Evaluating methods using samples of known ancestry

At the full suite of 17 loci, all 24 of the 100% red wolf pups were classified as pure red wolves by all three criteria. Ancestry estimates for the hybrids, however, were variable depending upon the ancestry of the mother (Table S1, Supporting information). Nine of 10 hybrid pups that had a 100% red wolf mother were classified as red wolf-coyote hybrids using all three criteria. One pup (11517-4) was classified as a hybrid by the BAPS and STRUCTURE-relaxed criteria; but not by the STRUCTURE-conservative criteria.

For the hybrid puppies with admixed mothers, the results were much more variable. The puppy with a 93.75% red wolf mother was classified as a hybrid by the BAPS and STRUCTURE-relaxed criteria, but STRUCTURE-conservative classified it as a red wolf (Table S1). The six puppies with the 96.85% red wolf mother were all classified as coyotes by both BAPS and STRUCTURE-conservative criteria (Table 2). Five of these individuals were classified as hybrids using the STRUCTURE-relaxed criteria: one would have been considered a coyote and all six had coyote *q* values >0.7.

The use of six loci had no impact on the classification of the pure red wolf puppies. All 24 of 100% red wolf pups were still classified as pure with all three criteria using just six loci (Table S2, Supporting information). The average absolute difference in the red wolf and coyotes *q* values produced by STRUCTURE at six versus 17 loci was very small (0.019 ± 0.015 SD). The results for the 17 hybrids were once again much more variable. BAPS classified all but three hybrid puppies as pure individuals: 13 were classified as coyotes and one as a red wolf (Table S2). Only one of these puppies was classified as hybrid by the STRUCTURE-conservation criterion: this individual was also classified as admixed by BAPS. Eleven pups would have been classified as coyotes and two as red wolves using this criterion. All hybrid puppies were classified as admixed using the STRUCTURE-relaxed criterion with six loci, although five individuals had coyote *q* values >0.7. The average absolute difference in STRUCTURE *q* values at 6 versus 17 loci for the hybrids was 0.143 with a standard deviation of 0.095.

Discussion

Red wolf ancestry

In contrast to our expectations, we found no pure red wolves and little evidence of red wolf introgression into the coyote population outside of the NEP. Three out of 82 individuals showed evidence of red wolf ancestry in the mitochondrial or nuclear genomes using the conservative criteria, although this number increased to ten under more a more relaxed criterion. The lack of a prominent red wolf genetic signature outside the NEP could be due to two processes. First, successful red wolf dispersal could be much more limited than we originally hypothesized, potentially due to high mortality rates. Gunshot mortality and vehicle collisions contribute to 36% of the annual mortality reported for red

wolves within the NEP (USFWS 2007). Lower survival rates for dispersing individuals have been reported for other wolf populations (Pletscher *et al.* 1997; Fuller *et al.* 2003). Second, the number of wolves that disperse is likely very small compared to the resident coyote population in this region. Given that the average age of reproduction of red wolves is about four years, at least five generations of red wolves have had the opportunity to intermix with coyotes since the first releases, providing ample time for introgression to occur. Because the population sizes of the two species are so disparate, any red wolf introgression into the coyote population is likely swamped and undetectable in nDNA within a few generations. This is supported by the fact that we detected one individual with the red wolf mtDNA haplotype that were classified as a pure coyote using nDNA analyses.

It is important to acknowledge that our inability to detect pure red wolves during our scat surveys does not mean that there are no wolves beyond the NEP. This survey took place during a limited 2-month period and sampling was diffuse across the landscape. Using a rough estimation based upon the number of roads in each grid cell we sampled, we surveyed less than 1% of the dirt roads in our study area. Nevertheless, our results suggest that red wolves and red wolf/coyote hybrids are a minor component of the canid population in this landscape. Adams *et al.* (2007) detected 93% of known resident red wolves when conducting a scat survey over 682 km of roads within the NEP, suggesting that road sampling is an effective survey method that is not biased against red wolves.

The lack of observed red wolf colonization and introgression beyond the NEP has several implications for the future management of this species. First, it raises questions about the fate of dispersing red wolves and whether mortality is limiting colonization of this species. Also, because red wolf introgression into the coyote gene pool outside the NEP was limited, it seems likely that any hybrid individuals detected within the NEP were born there as opposed to being produced outside the management area and dispersing back in. Thus, introgression into the red wolf gene pool is likely to occur from hybrids produced within the NEP, and management should continue to focus on preventing hybridization within the NEP. Despite 20 years of population growth and expansion within the NEP, the red wolf has not successfully colonized areas adjacent to the NEP in the absence of active management. Future reintroduction efforts in areas occupied by coyotes, which includes the entire historic red wolf range, would likely require intensive management efforts to curb hybridization as practiced in the NEP (Stoskopf *et al.* 2005).

Challenges of detecting hybridization and introgression

Our ability to accurately characterize hybridization in this canid population is limited by the incongruent results produced by the multiple admixture tests. Relying on traditional measures of statistical significance (i.e. BAPS and STRUCTURE-conservative) would suggest that only a small proportion of the canid population shows considerable levels of admixture (~5%). However, relying solely on STRUCTURE q values and a threshold of $q > 0.10$, a common practice in hybridization studies (Vaha & Primmer 2006; Sanz *et al.* 2009), suggests that 45% of the population is composed of hybrids. Our results from the test of the known hybrid puppies using seventeen and six loci suggest that this criterion is more likely to accurately detect hybridization, and no known red wolves were likely misclassified as hybrids using this criterion. Obviously, these different criteria for assessing admixture suggest dramatically different patterns of hybridization between sympatric canids in this region and highlight the importance of choosing appropriate criteria. However, the ability to detect significant levels of admixture is influenced by a variety of factors, especially the number of loci genotyped, degree of differentiation between genetic groups, and the use of training sets (Hauser *et al.* 2006; Latch *et al.* 2006; Vaha & Primmer 2006; Sanz *et al.* 2009). Because our genotypes were obtained via NGS, it was difficult to amplify the full suite of 17 loci and the lack of power at a reduced number of loci limited our ability to definitely assign ancestry to some individual samples particularly using the more conservative criteria.

Most studies typically consider an individual admixed by some subjective threshold of STRUCTURE q values, usually 0.1–0.2 (Vaha & Primmer 2006; but see Beaumont *et al.* 2001; Trigo *et al.* 2008). Although these cutoff values make interpretation of the results clear-cut, the biological and statistical significance of these q values is more difficult to assess (Pritchard *et al.* 2000; Corander & Marttinen 2006; Vaha & Primmer 2006). Adding Bayesian credibility intervals to these estimates helped evaluate the statistical significance of the q values we observed, although the results from the test of the puppy samples suggest these measures of admixture may be overly conservative.

Testing these programs using genotypes from puppies of known ancestry provided additional insights into the potential errors associated with these programs. All 100% pure red wolf puppies were classified as pure red wolves using all three criteria at both 17 and six loci: thus, we believe our failure to find any pure red wolves among our scat samples was unlikely to be caused by misclassifications by either program. The results from the hybrids, however, suggest these pro-

grams can produce variable estimates of ancestry for admixed individuals. Red wolf *q*-values from STRUCTURE for individuals with ~50% red wolf ancestry ranged from 0.07 to 0.551 using 17 loci. At 17 loci, all hybrids except those with <100% red wolf mothers were classified as admixed using all three criteria. However, at six loci most of the hybrids were classified as pure individuals using BAPS and STRUCTURE-conservative, sometimes as different species, even though they had similar ancestral backgrounds and in some case the same parents. This suggests that BAPS and the STRUCTURE credibility intervals are both strict measures of admixture and the inability to detect introgression may be due to a lack of power in the dataset. Relying solely on the STRUCTURE *q* values would have provided a more accurate picture of the ancestry of these individuals.

Q-values even showed variability among related individuals that shared parents. The puppies of female 11429 are of particular interest because the results of the Bayesian analyses would strongly suggest these individuals are coyotes. Female 11429 is a descendent of the hybrid male that backcrossed with the red wolf population and still carries a coyote private allele that was inherited from this hybrid. Thus, it appears even the relative little contribution of coyote ancestry from the red wolf mother affected the performance of the programs. The variability in *q* values associated with animals of similar ancestry suggests that we may have underestimated the true amount of admixture in the wild canid population. Clearly, more extensive tests are necessary to evaluate the performance of these programs in detecting hybridization.

Sequencing mtDNA provided another interesting component to our assessment of admixture. Several 'pure' individuals possessed haplotypes assigned to other species, suggesting evidence of introgression from nuclear markers has been eroded due to backcrossing. This is especially relevant because an apparently 'pure' coyote had the red wolf mtDNA haplotype, suggesting that this haplotype introgressed into the local coyote population many (>4) generations ago. An alternative explanation is that the red wolf-specific haplotype was historically present in the coyote population. However, several studies have sequenced the mtDNA control region from coyotes in Canada (Wilson *et al*. 2000), the southeastern US (Adams *et al*. 2003b), and the historic zone of sympatry for red wolves and coyotes in Texas, USA (Hailer & Leonard 2008) and none have found the red wolf haplotype in any of those coyote populations. Given the close proximity of this individual to the NEP, it seems more likely that it is the result of red wolf introgression. Omitting collection of mtDNA sequence data would have underestimated the prevalence of historic hybridization and the degree of backcrossing. Conversely, by relying solely on mtDNA sequencing, we would have erroneously concluded that there were pure red wolves outside the NEP and we could not have detected admixed individuals. Based on the patterns we observed in this study, we highly recommend that researchers assessing hybridization use multiple genetic measures to evaluate admixture and be aware of the limitations of each method.

Canid admixture

Despite the challenges associated with interpreting these results, several conclusions can be drawn about this canid population. First, it is evident that admixture between multiple *Canis* species is occurring in this region. At least two individuals (Canids 10 and 59) had ancestry assignments indicative of F1 coyote/dog hybrids and two others (Canids 55 and 81) appeared to be F1 coyote/dog × dog backcrosses. To our knowledge, these are the first reports of such hybrids detected using nuclear DNA data. Studies based on morphological characters suggested that coyotes hybridized extensively with domestic dogs (Gipson *et al*. 1974; Freeman & Shaw 1979) as they first colonized the eastern US, but the frequency of this occurrence, especially in modern times, has not been evaluated using genetic methods. Mengel (1971) predicted based on a captive breeding study that shifts in breeding cycles for F1 hybrids and physical deformities would limit introgression of dog genotypes into coyote populations. However, the present study and a mtDNA study by Adams *et al*. (2003b) documented the presence of dog and coyote mtDNA haplotypes in 'pure' individuals of the other species, suggesting that these hybrids are capable of backcrossing with the parental species.

There is also evidence of admixture with grey wolves in this coyote population. There are several hypotheses that could explain the presence of grey wolf genetic material in this canid population. Grey wolves have tremendous dispersal capabilities and individual wolves have been known to travel several hundred to thousands of kilometers in search of resources (Mech & Boitani 2003). The nearest populations of grey wolves are 1 500 km away in eastern Canada (Wilson *et al*. 2000) and grey wolves and grey wolf/eastern wolf (*C. lycaon*) hybrids have been reported in the northeastern US. Individuals with grey wolf genetic ancestry could have dispersed into this region from Canada; however, given the distance needed for a grey wolf to reach this region while avoiding potential sources of mortality, this scenario seems unlikely. More plausible is that the ancestry of these individuals traces to escaped wolf or wolf/dog hybrid pets or their descendants. It is virtually impossible to estimates the number of wolf–dog hybrids in the

US, but reports suggested the population was around 300 000 during the 1990s (Willems 1995).

Such findings have implications not only for future red wolf recovery but also for management of other canid species. Hybridization between canids is often cited as a process that is most pronounced in small populations when Allee effects are expected to be most common (McCarley 1962; Allendorf *et al.* 2001; Wayne & Vila 2003; Grewal *et al.* 2004; Munoz-Fuentes *et al.* 2009). However, coyotes in eastern North Carolina appear to be admixing with multiple canid species despite their large population size and widespread occurrence. Thus, hybridization may not be a process limited to small populations. Coyotes are heavily harvested in this region, which may disrupt stable social units and encourage hybridization as has been found in eastern wolves (*C. lycaon*, Rutledge *et al.* 2010). Observing admixture between these abundant canid species raises questions about the mechanisms that influence the hybridization process.

The increasing documentation of hybridization among *Canis* species has important implications for carnivore biology and management. Coyotes in the northeastern U.S. show evidence of mixed ancestry with eastern wolves, likely due to historic interbreeding during the coyote expansion eastward (Wilson *et al.* 2000; Kyle *et al.* 2006; Kays *et al.* 2010). Wolves in the Great Lakes region appear to be the product of mixing between grey wolves and eastern wolves (Wilson *et al.* 2000; Kyle *et al.* 2006; Leonard & Wayne 2008; Wheeldon & White 2008), with a potential gradient of ancestry stretching across the contact zone for these species (Mech & Paul 2008). Morphological and genetic data suggest that stable hybrid zones may have existed in the central US where multiple species historically existed in sympatry (Nowak 2003; Hailer & Leonard 2008). Eastern North Carolina may be even more complex than these systems, since there are genetic inputs into the canid population from four species, including one endangered species (red wolf), one abundant non-native species (coyote), one domestic species (dog), and one species whose nearest population is 1 500 km from this area (grey wolf). Given the dominant role large canids play in ecological systems, questions arise over the ecological role assumed by hybrids. Further research regarding the ecological traits of these species and their hybrids is paramount to understanding this ecosystem, including further surveys to gain a better representation of canid distribution across this region.

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J. H. B. is a PhD candidate at the University of Idaho studying hybridization between red wolves and coyotes. His research focuses on understanding extent of hybridization between these species and linking evaluations of hybridization with management of endangered species. L. P. W. is a professor at the University of Idaho and member of the Red Wolf Recovery Implementation Recovery Team. Her research focuses on the molecular ecology and conservation genetics of a variety of animal taxa.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 BAPS and STRUCTURE ancestry coefficients for the 17 known hybrid puppies (~50% red wolf) and the ancestry of their mothers at the full suite of 17 loci. Values shaded in gray indicate STRUCTURE q-values for which the credibility interval does not overlap 0 and thus provides statistical support for genetic ancestry from this species. Individuals with identical numbers for the first five digits are littermates.

Table S2 BAPS and STRUCTURE ancestry coefficients for the 17 known hybrid puppies (~50% red wolf) and the ancestry of their mothers at the six most amplified loci. Values shaded in gray indicate STRUCTURE q-values for which the credibility interval does not overlap 0 and thus provides statistical support for genetic ancestry from this species. Individuals with identical numbers for the first five digits are littermates.

Table S3 Mitochondrial DNA sequences identified from scats and their associated GenBank Accession numbers. The first column indicates the taxonomic identification of each sequence at the genus/family level.

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