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The use of museum specimens to reconstruct the genetic variability and relationships of extinct populations

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Abstract. In this review, we discuss the use of DNA from museum specimens to address conservation genetic questions. We provide four examples from our previous studies of the northern hairy-nosed wombat, African wild dog, Ethiopian wolf and red wolf. These species were genetically surveyed using two molecular approaches: first, analysis of short sequences in the mitochondrial genome using species-specific primers, and second, analysis of hypervariable microsatellite loci. The studies demonstrate that museum-derived DNA adds an important dimension to the genetic study of extant populations. Inaccessible populations can be studied, and both the loss of genetic variation and its distribution over space and time can be better understood. Finally, analysis of additional museum material provides definitive evidence for a hybrid origin of the red wolf.

Key words. Ancient DNA; conservation genetics; mtDNA; microsatellites.

Introduction

Recent advances in molecular biology have allowed the routine extraction and analysis of DNA from historic or ancient tissue specimens[18,41,44]. This breakthrough may allow direct measurement of temporal changes in allele frequencies and gene coalescence that presently can only be inferred from the spatial distribution of alleles in extant populations[1,22]. The ability to observe the past genetic structure of extant populations is of great importance to conservation geneticists who wish to understand the genetic effects of population bottlenecks or to reconstruct past population substructure[2,4,24]. Additionally, the use of museum material allows access to samples that may be otherwise impossible to obtain. Finally, knowledge of phylogenetic relationships and historical biogeography of taxa is often confused due to limited genetic information from extinct close relatives. Museum material may allow the testing of phylogenetic and biogeographic hypotheses predicted from the analysis of extant populations.

In this report we review our recent DNA studies using museum specimens that exemplify their use for the purposes discussed above (table 1). We describe how analysis of museum material allows a direct estimate of the decline in genetic variability experienced by Australia's most endangered mammal, the nothern hairy-nosed wombat[7,40]. We demonstrate that the single remaining extant population is genetically very similar to one several thousand kilometers distant and thought to be conspecific. Similarly, we uncover the relationships of extant populations of Simien jackals and African wild dogs to extinct or inaccessible populations that are only represented in museum collections. Finally, we clarify the origin of the red wolf, a species that has hybridized extensively with two conspecifics in the recent past[44].

The approaches we have used on museum material reflect the fact that DNA extracted from historic tissue samples is often highly degraded due to the methods used for tissue storage at the time of collection and the fractionation of DNA that occurs with time[42]. For this reason, PCR amplification of short sequences that have high copy number, such as those found in the mitochondrial DNA genome, is often the aim of studies on museum specimens[41,36,37,44]. More recently, single locus nuclear microsatellite DNA has also been successfully amplified from historic samples. These sequences belong to a class of hypervariable simple sequence repeats that are useful for assessing differentiation at the population level as they are highly polymorphic[9,21,29,38,39]. These regions have been used successfully to assess levels of hybridization in the red wolf[33,34] and we discuss their use in the analysis of historic samples.

The genetic structure and conservation of the Ethiopian wolf

With fewer than 500 individuals left, the Ethiopian wolf (*Canis simensis*), more commonly known as the Simien jackal, is regarded as the most highly endangered canid[13]. The species is restricted to the fertile highlands of Ethiopia, over 3000 m, where parkland is being converted for use by local farmers. As a result, Ethiopian wolf populations have been declining due to loss of habitat and human activities at least since the last century[25,47]. Even over the past 10,000 years, Ethiopian wolf population sizes may have been progressively decreasing. The afro-alpine habitat of Ethiopian wolves was approximately 50 times larger than its present size of 2275 km^2 during the last glacial period (10,000–70,000 years ago) when the African tropics were generally cooler and drier than today[5,16,19,46]. Reduced levels

Table 1.

Species	Problem	DNA region analyzed	Conclusions	References
Ethiopian wolf	Genetic structure of disjunct populations	mtDNA control region	Northern population 1.5% divergent from southern population, suggesting significant divergence.	17
African wild dog	Genetic structure of living and historic populations	mtDNA control region	A distinct west African genotype identified, loss of genotypes documented in a Transvaal population and past genetic associations of southern African dogs defined.	14
Northern hairy-nosed wombat	Effects of a bottleneck on genetic variability	mtDNA cyt b, nuclear microsatellite analysis	This species has lost up to 60% heterozygosity compared to the southern species. Effective population size estimate of 10–20 during past 120 years.	40
Red wolf	Evolutionary origin	mtDNA cyt b, nuclear microsatellite analysis	Both types of data are consistent with the red wolf having a hybrid origin through interbreeding of coyotes and gray wolves.	33, 34

of genetic variability have been found in Ethiopian wolves[17], and in at least one population, the Ethiopian wolves are sympatric with domestic dogs and hybridize with them. Conservation efforts for this species have been delayed, in part, because of a lack of information regarding the genetic structure of the Ethiopian wolf populations[15,16].

A study of extant populations from the Sanetti Plateau and Web Valley in the Bale Mountains region was initiated in 1988[15,16] and a genetic analysis began in 1990[17]. A molecular genetic analysis found no polymorphism in 400 bp of the mtDNA control region of individuals from both the Sanetti Plateau and Web Valley populations[17]. In addition, analysis of 9 microsatellite loci showed levels of genetic variability in Ethiopian wolves to be considerably lower than those of other large canids and found domestic dog alleles in the Web Valley population, an area where domestic dogs were also present. However, the two study populations represent only a small fraction of the geographic range of the species and northern populations, across the Rift Valley, were suspected to be highly differentiated from their southern conspecifics. Because of national unrest, samples from the northern populations could only be obtained from museum skins.

We isolated DNA from two skins from the northern population and were able to amplify 134 bp of the mtDNA control region. These sequences were compared to those from the Web and Sanetti populations. Our preliminary results show that both individuals have the same sequence, which differs from those sequences found in individuals south of the Rift valley by 2 bp (1.5%, fig. 1). This differentiation is not surprising since the two populations have not been connected since the last glacial period (10,000–70,000 years ago). If significant differentiation is confirmed by analysis of additional individuals and more control region sequence, then the two populations should, if feasible, have separate captive breeding programs. In addition, the northern populations may provide a source of Ethiopian wolves that have not interbred with domestic dogs.

The effect of bottlenecks in the northern hairy-nosed wombat

The northern hairy-nosed wombat (*Lasiorhinus krefftii*) experienced a dramatic decline in population size beginning approximately 120 years ago with the arrival of European settlers to North Queensland, Australia. It once had a geographic range of several thousand km^2, but by 1981 only 20–30 of the large burrowing marsupials remained in a restricted colony occupying less than 100 km^2 of Epping Forest near Clermont, Central Queensland[7,8]. Today the colony is protected and the population has increased to around 70 individuals. Sequence analysis of the mtDNA cytochrome b gene along with microsatellite analysis[40] was used to examine phylogenetic relationships and the level of genetic variation in the extant Epping Forest population compared to that of two populations of southern hairy-nosed wombats (*Lasiorhinus latifrons*). An important intraspecific comparison was provided by analysis of DNA from museum skins of an extinct population of northern hairy-nosed wombats near Deniliquin, New South Wales. The skins were collected before 1910 and at a locality about 1000 km from Epping Forest. These pu-

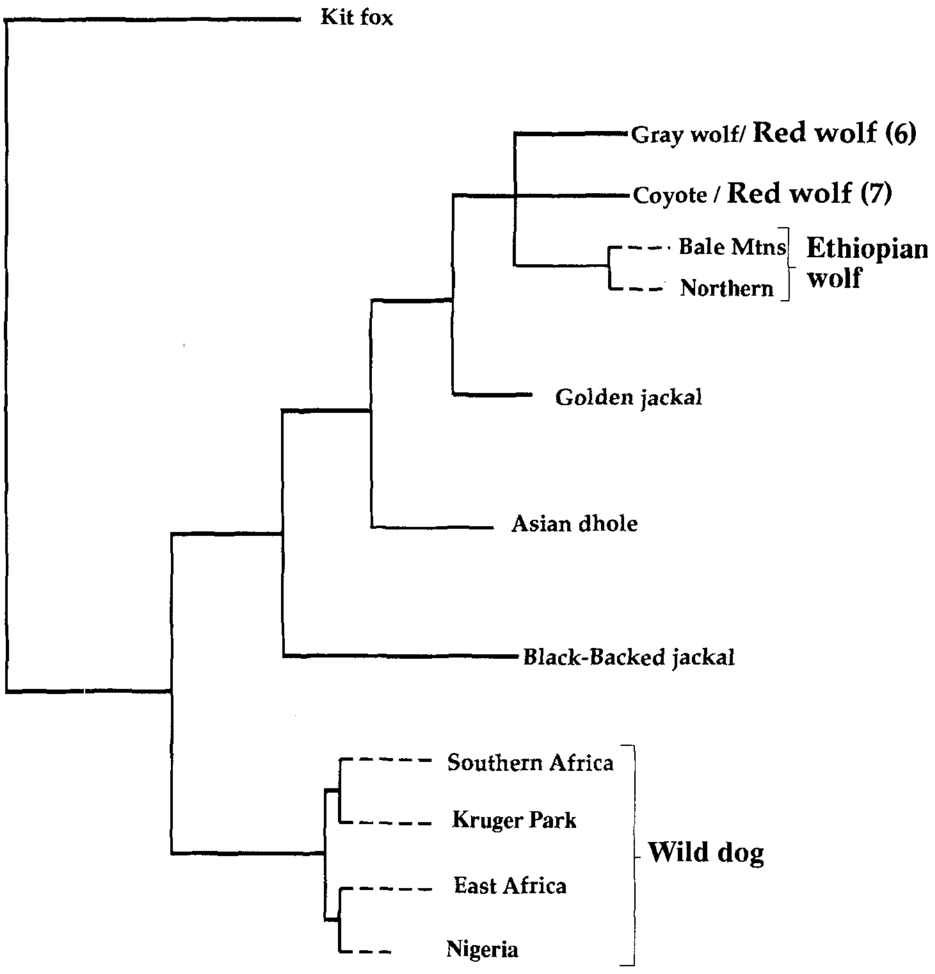

Figure 1. Phylogeny of the Canidae based on 2.1 kb of mtDNA sequence. Placement of red wolf genotypes from 13 museum specimens based on 250–400 bp of cytochrome b sequence is indicated. Wild dog (*Lycaon pictus*) and Ethiopian wolf (*Canis simensis*) genotypes are based on 134 bp of control region sequence and are indicated by dashed lines.

tative outbred populations provided a reference for levels of genetic variability likely to be found in the pre-bottlenecked Epping Forest wombats.

Taylor et al.[40] first confirmed the species identity of museum skins from the Deniliquin northern hairy-nosed wombat population by using a 90 bp region of the cytochrome b gene. This region is diagnostic because it contains three unique base pair changes that distinguish the Epping Forest northern-hairy-nosed wombat from the southern hairy-nosed wombat. In addition, Taylor et al.[40] found that the museum specimens shared a diagnostic simple sequence allele with the extant northern hairy-nosed wombat population. Finally, genetic distance measures based on 8 simple sequence loci cluster the two northern hairy-nosed wombat populations together (fig. 2). Levels of polymorphism and heterozygosity in the Epping Forest population were significantly lower than the southern

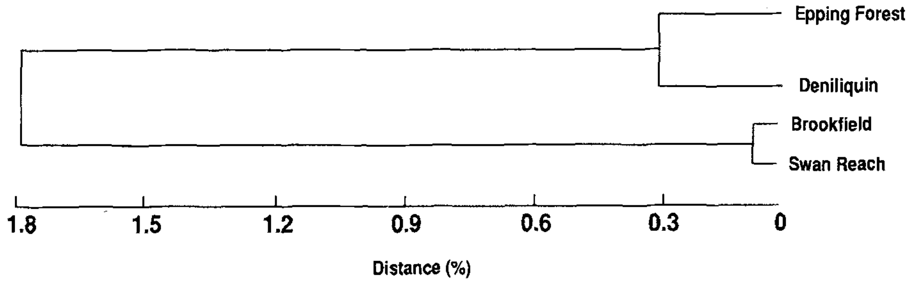

Figure 2. Cluster analysis of allele frequencies of 8 microsatellite loci in northern hairy-nosed wombats (Epping Forest and Deniliquin) and in southern hairy-nosed wombats (Brookfield and Swan Reach)[40].

hairy-nosed wombat populations and marginally lower than those in the extinct Deniliquin population. This result suggested that the Epping Forest wombats had lost 40% to 70% of their heterozygosity during the past 120 years, a decline consistent with an effective population size of only 10–20 individuals.

The genetic structure of the African wild dog

The African wild dog (*Lycaon pictus*) has the highest prey capture success of any large African carnivore[10,12,35]. It once ranged over most of Africa south of the Sahara, in areas of dry woodland and savannah. However, due to habitat change and disease, many populations have vanished or been severely reduced in number. As a result, the present populations are highly fragmented and contain no more than several thousand individuals[11,13]. The most severe losses have occurred in western and eastern Africa with populations in southern Africa currently stabilized in protected areas[11]. However, most captive populations are thought to be of southern African origin, and consequently do not provide the appropriate stock for reintroduction to regions of eastern and western Africa where wild dogs have disappeared. Furthermore, although populations in southern Africa may currently be stabilized, many are isolated and probably retain low levels of genetic variability, thus lending themselves susceptible to future environmental catastrophes[14,28,32].

Over 100 African wild dogs from populations in eastern (Kenya and Tanzania) and southern Africa (Zimbabwe and the Republic of South Africa), were examined for mtDNA restriction fragment length polymorphisms (RFLP) and variation in cytochrome b sequence[14]. The results show that about 1% sequence divergence exists between southern and eastern populations corresponding to two distinct monophyletic groups, each containing three RFLP genotypes (fig. 1). Based on these results, eastern and southern populations were given separate subspecific designations[3]. Furthermore, whereas other populations had several mtDNA genotypes, a population in Kruger Park in the Transvaal region of the Republic of South Africa was found to have only a single genotype. We hypothesized that decreased levels of variation in the Kruger Park population reflected either a population bottleneck that occurred about 1900 due to war and disease or simply reflected the stochastic loss of genotypes expected in such a small population if it was completely isolated[14,45].

To investigate the relationships of inaccessible western African wild dogs and to understand the temporal changes in genotype frequencies of declining populations, DNA was extracted from museum skins and 140 bp of mtDNA control region was sequenced. Samples from central and western Africa were obtained as well as historic samples from Malawi and other populations in southern Africa which existed before and after the onset of the proposed bottleneck in the Transvaal region. Higher levels of heterozygosity and increased genetic polymorphism in the pre-1900 populations would support theories of a recent population bottleneck in the Transvaal region.

Preliminary analysis of 140 base pairs of the control region sequence provides several interesting insights into the genetic structure of African wild dogs. First, a museum skin collected from Nigeria has a distinct genotype, showing on average about 4% sequence divergence from eastern African wild dogs and 6–7% sequence divergence from wild dogs of southern Africa. This level of differentiation is equivalent to that between the long separated eastern and southern wild dog populations. Second, analysis of two specimens collected from Malawi indicated two genotypes common in southern Africa, one of which is a genotype found in Kruger Park (fig. 1). These results suggest that at least in the 1890s when these samples were collected, wild dogs in Malawi, which borders Tanzania to the north, were part of a large southern African population that extended from Malawi to the Republic of South Africa. Finally, of the four skins collected from the Transvaal region of the Republic of South Africa, three had genotypes commonly found in this same region today. The fourth had a genotype no longer found in this region but common in Botswana, Zimbabwe, and found in a Malawian skin. However, this individual was collected in 1938, well after the proposed bottleneck, and hence our result suggests that the loss of genotypes due to drift is not counterbalanced by migration. More historic samples need to be analyzed, but our hypothesis is that the Kruger Park population, although stable, is extremely isolated and is not receiving gene flow from other southern African populations because of fences and habitat barriers surrounding the park.

The red wolf: a distinct species or hybrid?

The red wolf, a medium sized North American canid, was once common in the south-eastern United States[6]. Because of predator control measures and habitat changes that began with the arrival of European settlers in the 19th century, the red wolf was extinct in the wild by about 1975. Alteration of its habitat and reduction in numbers after the 1930s led to hybridization between red wolves and coyotes[26], and by the 1960s pure red wolves had nearly disappeared except for a small population in south-eastern Texas. Fourteen individuals thought to be pure red wolves were selected from this population as founding stock for a captive breeding program. Captive-bred red wolves have been experimentally released in Alligator River National Wildlife Refuge in eastern North Carolina to re-establish wild red wolf populations.

Recently, debate concerning the taxonomic status of the red wolf has focused on the influence of interspecific hybridization on its genetic composition[20,23,27,31,44]. Morphologic analysis of pre-1940 red wolves showed them to be a distinct form intermediate between gray wolves and coyotes[27]. However, analysis of restriction site and cytochrome b sequence data showed that red wolves from the captive colony, the 1974–1976 source population and six museum skins collected before 1930 had genotypes identical or similar to those found in gray wolves or coyotes. No phylogenetically distinct mtDNA genotypes were found as might be expected if the red wolf was a distinct species[44]. The results were consistent with a hybrid origin of the red wolf. However, the red wolf genotype may have been missed in the limited sample and analysis of nuclear genes might have revealed a higher level of distinction.

Consequently we analyzed 10 microsatellite loci in 30 individuals from the captive colony of red wolves, 150 gray wolves and 150 coyotes. Our results showed that red wolf allele frequencies were intermediate but closer to those of coyote populations than populations of gray wolf[33]. Additionally, although many unique alleles were observed in gray wolves and coyotes, very few were found in red wolves. All alleles found in red wolves were also present in coyotes[33]. This evidence led us to conclude that the captive colony was descended from ancestors which had extensively hybridized with coyotes. We also analyzed 300 bp of cytochrome b sequence from 7 additional red wolves collected between 1905–1930 before hybridization was thought to be significant. As before, their sequences were very similar or identical to either gray wolf or coyote genotypes[34,44].

Finally, Roy et al.[33,34] analyzed 10 nuclear microsatellite loci from DNA extracted from 16 red wolf skins collected before 1930. Nei's genetic distance between the historic red wolves and non-hybridizing gray wolves was 0.393, a figure similar to the mean intraspecific distance between gray wolf populations (0.315) (table 2). Likewise, the genetic distance between historic red wolves and coyotes was similar to the mean distance between coyote populations (0.245 and 0.254 respectively). In contrast, mean distance values between non-hybridizing gray wolves and coyotes was much higher (0.6) (table 2). A phylogenetic hypothesis consistent with these results is a hybrid origin of the red wolf through interbreeding of coyotes and gray wolves. This result does not support the theory, based on fossil evidence, that the red wolf is the ancient predecessor of both coyotes and gray wolves[27]. If this were true, coyotes and gray wolves should be more closely related to each other than to the red wolf.

In conclusion, we have reviewed examples of the use of museum specimens to address conservation genetic questions. Molecular genetic analysis of DNA isolated from museum specimens provides a more precise estimate of past levels of genetic variability in declining populations and may allow a reconstruction of the genetic subdivisons existing within species. Populations that are inaccessible may also be analyzed and targeted for conservation efforts if they are found to be distinct. Moreover, a more complete knowledge of the population genetic substructure within species can potentially be obtained using museum collections, allowing for better genetic management of endangered species. Finally, the historical influences of gene flow, changes in

Table 2. Nei's genetic distance estimates from allele frequencies of 10 microsatellite loci.

	Alaskan wolves	Alberta wolves	S. Quebec wolves	Alaskan coyotes	Maine coyotes	Californian coyotes	Captive red wolves	Historic red wolves	Golden jackal
Alaskan wolves	0.000								
Alberta wolves	0.259	0.000							
S. Quebec wolves	0.327	0.359	0.000						
Alaskan coyotes	0.631	0.636	0.393	0.000					
Maine coyotes	0.579	0.629	0.297	0.282	0.000				
Californian coyotes	0.574	0.570	0.319	0.243	0.237	0.000			
Captive red wolves	**0.700**	**0.641**	**0.302**	**0.407**	**0.368**	**0.302**	**0.000**		
Historic red wolves	**0.368**	**0.418**	**0.282**	**0.290**	**0.249**	**0.196**	**0.303**	**0.000**	
Golden jackal	0.875	1.262	1.142	1.809	1.234	1.339	1.486	1.310	0.000

Populations of North American gray wolf, coyote and red wolf were compared. The golden jackal was included as an outgroup[33,34]. Bold numbers are distances between red wolves and other wolf-like canids.

population size, and hybridization on the genetic composition of endangered species can be directly observed by analysis of historic populations. In well-sampled species, we may have the opportunity to follow directly temporal changes in gene frequency that in the past could only be inferred from spatial changes in allele frequencies[41].

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