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Effects of fragmentation and isolation on genetic variability of the Italian populations of wolf *Canis lupus* and brown bear *Ursus arctos*

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During the last centuries many West European populations of wolf *Canis lupus* Linnaeus, 1758 and brown bear *Ursus arctos* Linnaeus, 1758 have been extirpated from most of their former ranges. Isolated populations of wolves (about 300 – 400 animals) and brown bears (about 80 – 100 animals) actually survive in the Italian Apennines, while very few (5 – 10) brown bears remain in the Italian eastern Alps. We have investigated the consequences of isolation, demographic decline, and random drift on genetic variability of the Italian populations of wolf and brown bear using restriction site analysis and nucleotide sequencing of portions of the mitochondrial genome. The studied sequences were homogeneous within-populations of both species, but there was a fixed difference in mtDNA between brown bears form the Alps and from the Apennines. Random drift since the time of isolation is a plausible explanation for both results. These findings suggest that wolves and bears have small effective population sizes and, thus, they will continue to loose genetic variability by random drift in the near future. Conservation efforts should be directed towards an increase of the annual growth rates of these populations. The individualization of discrete phylogeographic units in the brown bear suggests to manage them separately in order to preserve the existing gene diversity among populations.

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Introduction

During the last few centuries the growth of the human population and the transformation of natural habitats have had dramatic impacts on wildlife, and particularly on large mammals. Deforestation, the spreading of agriculture, and hunting pressure on ungulates and predators caused a process of gradual but persisting fragmentation, isolation and local eradication of populations of several species. Some two hundred years ago, the wolf *Canis lupus* Linnaeus, 1758 and the brown bear *Ursus arctos* Linnaeus, 1758 were present in large numbers in most parts of Europe (Zimen and Boitani 1979, Sørensen 1990). Their populations were large and, as a consequence of wide dispersal ranges, local populations were

connected by migrants. Therefore, gene flow could have been high enough to maintain a large effective population size both in the wolf and the brown bear.

The process of contraction of the range of the brown bear started already in ancient times. The species disappeared from Great Britain about 800 – 1000 years ago and since that time local extinctions of west European brown bear populations occurred continuously. Today only a few small and isolated populations of the brown bear survive in Spain (Cantabrian mountains), in France (Pyrenees), in Italy (eastern Alps and central Apennines) (Sørensen 1990). The Italian brown bears are completely isolated from the larger Balkan brown bear population, although a trend for an expansion of the Slovenian brown bear population towards the eastern Italian Alps has been reported very recently (F. Perco, pers. comm.).

The eradication of the wolf from central and western Europe was a much more recent, but perhaps most rapid process. Extermination of wolves was dramatic during the 19th century, and continued at least until World War II (Zimen and Boitani 1979). Today the wolf is extinct in central and northern Europe, and relatively small, isolated populations survive in Portugal, Spain, and Italy (northern-central Apennines). The Italian wolves are isolated from the larger Balkan populations since the end of 1800, when all the local Alpine wolf isolates were exterminated by direct human persecution (Cagnolaro *et al.* 1974).

Declining numbers of individuals, fragmentation, isolation, and extinction of local stocks can be predicted to have important consequences on the genetic make-up of the surviving populations (Gilpin 1987). The genetic effects of fragmentation have been studied in a few cases in natural populations (Templeton *et al.* 1990), while we have more information documenting the consequences of bottlenecking and quasi-extinction on the residual genetic diversity of many endangered species (Lacy, in press). Historical processes of population decline, fragmentation, and isolation can be considered unfortunate experiments from which we can learn how to manage and conserve relic populations and endangered species, and how to cope with the worldwide erosion of genetic diversity. The recent development of molecular genetic analyses applied to natural populations (Hoelzel and Dover 1991) made it possible to expand greatly the field of conservation genetics.

In this paper data on genetic diversity within and among Italian populations of wolf and brown bear, obtained by mitochondrial DNA (mtDNA) sequence analyses, are discussed in the light of available historical information on the decline, fragmentation and isolation of those populations. Implications for conservation and management of populations of the wolf and the brown bear are outlined.

Material and methods

Tissue (liver and heart) and blood samples were collected from 34 wolves (belonging to the population of the northern and central Apennines) and from 10 brown bears (belonging to the population of the central Apennines). Most of the wolves were killed illegally or accidentally, while

brown bears were captured for a project of radio-tracking. Moreover, we obtained hairs and excrements of two brown bears from the Alpine population (Adamello-Brenta Natural Park).

Total DNA was purified from blood or tissues using standard proteinase K digestion and phenol/ /chloroform/isoamyl alcohol extractions (Hillis *et al.* 1990), from hair roots using the Chelex (Bio Rad) procedure (Walsh *et al.* 1991), and from excrements follwing Hoss *et al.* (1992). Wolf mtDNA was analyzed using the restriction site method (Dowling *et al.* 1990). Aliquots of 10 μg of total DNA were digested with a set of 19 restriction endonucleases, DNA fragments were separated by agarose gel electrophoresis, capillary blotted to nylon membranes, and hybridized with a cloned entire mtDNA probe, which was labelled using the DIG-labelling procedure (Boehringer). Mitochondrial DNA restriction patterns were detected after 1-3 hours of exposition of autoradiographic Kodak paper. Bear mtDNA was studied using the polymerase chain reaction (PCR, Saiki *et al.* 1988) and direct sequencing of the amplified fragments (Sanger *et al.* 1977). Aliquots containing less than 100 ng of total DNA were used for double-strand PCR amplifications of portions of the mtDNA cytochrome b gene and of the control region (Brown 1985) by means of oligonucleotide primers designed by Kocher *et al.* (1989) and Hoss *et al.* (1992). Double-strand mtDNA fragments were sequenced using thermal cycling Sequenase (USB) and standard sequencing gel electrophoresis (Hillis *et al.* 1990).

Results

Using 19 6-base cutter restriction endonucleases for digesting the mtDNAs of 34 Italian wolves we obtained 60 restriction sites, corresponding to a survey of about 2% of the 16.800 bp mitochondrial genome of canids (Wayne *et al.* 1992).

The restriction patterns were identical between individual wolves (an example is given in Fig. 1). Therefore, restriction site analysis could not reveal any sequence variation within the assayed mtDNA samples of Italian wolf.

We amplified about 300 bp of the cytochrome b gene, and a 100 bp portion of the control region, corresponding to a survey of about 2% of the mtDNA of the

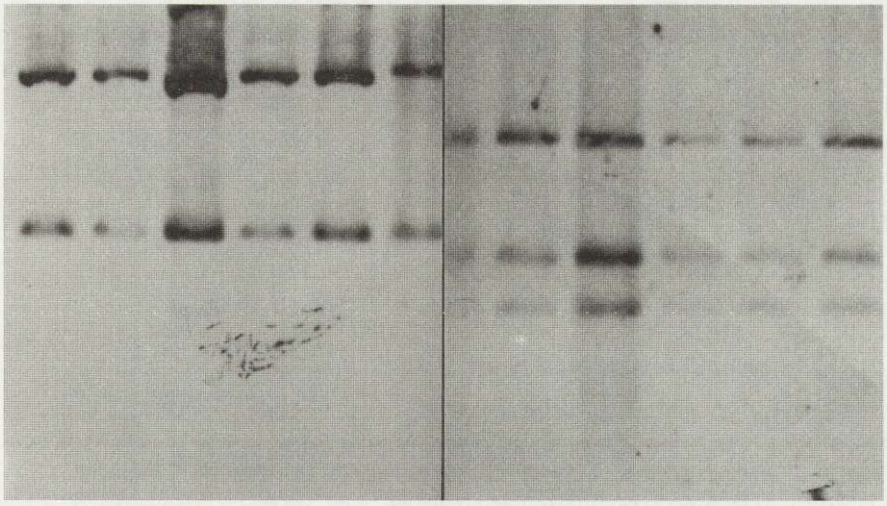

Fig. 1. Mitochondrial DNA restriction fragments obtained with endonuclease Xba I (left) and Hinf I (right) in the Italian wolf.

brown bear. Individual nucleotide sequences (an example is given in Fig. 2) were aligned and compared within and between populations. The sequences were identical among individuals of the same population, and there were 2 nucleotide substitutions (corresponding to an estimated per cent sequence divergence p = 0.005) between brown bears from the Alps and the Apennines. Thus, mtDNA sequence analysis could not reveal any nucleotide variation within the two Italian brown bear populations, but it revealed genetic divergence between them.

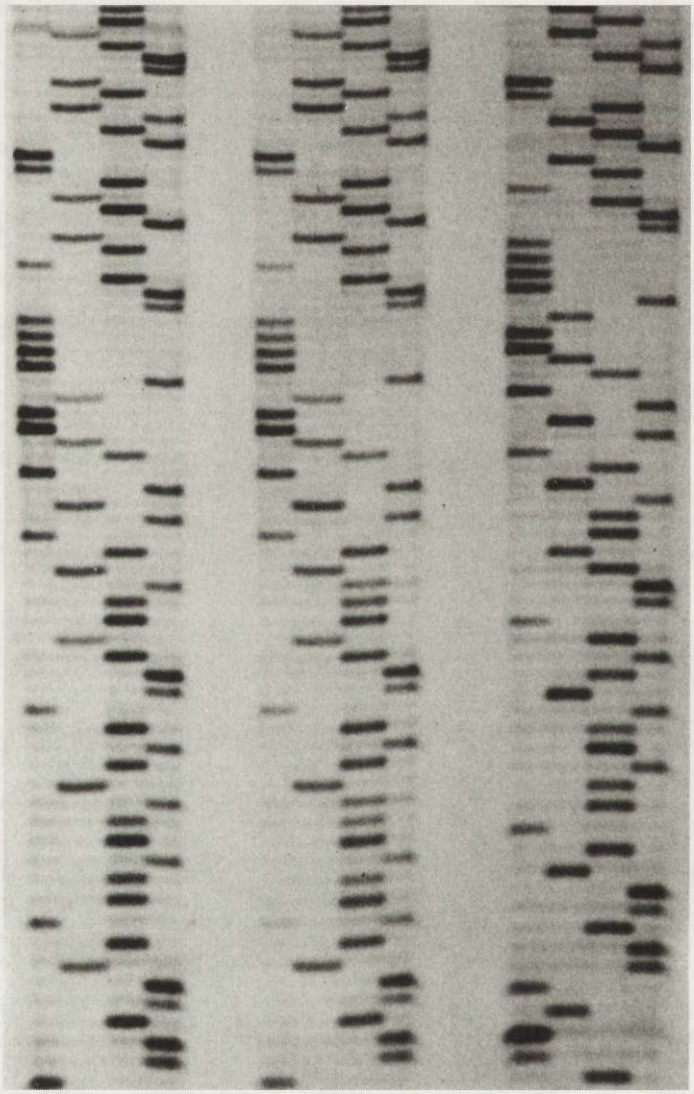

Fig. 2. Mitochondrial DNA cytochrome b sequences of three brown bears. Left and right: two brown bears from the Apennine population. Centre: a brown bear of North American origin. The nucleotide order is (from left): GATC.

Discussion

The Alpine wolf populations declined during all the 18th century until they were completely eradicated (Cagnolaro *et al.* 1974). Thus, the wolves in Italy are isolated since more than one century. The contraction of the range and the decline of the size of the peninsular wolf population continued at least until World War II. The information available on the present situation of the wolf in Italy suggests a recent inversion of the negative demographic trend. The distribution of the wolf is now extending and population sizes are increasing (Ciucci and Boitani 1991). According to several investigators the current population size amounts to about 300 to 400 wolves (Ciucci and Boitani 1991, Boscagli 1991).

Restriction fragment analysis revealed an absence of mtDNA variation in the 34 Italian wolves studied. This sample of individuals corresponds to about 11% – 15% of the average number of females being present per generation. Restriction site analyses cover only a small fraction (usually 2% – 5%) of the entire mtDNA, and probably underestimate nucleotide sequence divergence. Nevertheless, using this method 5 mtDNA haplotypes have been resolved in North American wolves, and 7 haplotypes in European and Asian specimens (Wayne *et al.* 1992). Therefore it is plausible that the Italian wolf population has low, if any, variation in mtDNA sequence.

Avise *et al.* (1984) stated that the rate of mtDNA coalescence in an isolated population at carrying capacity is related to its female effective population size (N_{ef}). A population has a high probability to retain a single mtDNA haplotype, i.e. to become monomorphic, after 4 N_{ef} generations since its isolation. Assuming that the Italian wolves are isolated since 100 – 150 years (corresponding to 50 – 75 generations, given a generation time of 2 years – Wayne *et al.* 1992), only a single mtDNA haplotype is likely to have survived if N_{ef} = 15 – 20. We have no data to obtain direct estimates of N_{ef} in the Italian or any other wolf population. However, we agree with Harris and Allendorf (1989), and with Mace and Lande (1992) that N_e is probably 1/5 – 1/4 of N_o (the observed population size) in many vertebrate species. This ratio has been considered by Boitani (1984) and by Wayne *et al.* (1992) in studies on wolves. In this case, an N_o of 300 – 400 corresponds to an N_e of 60 – 100, and to an N_{ef} of 30 – 50. Thus, it is possible that the Italian wolves have retained a single mtDNA lineage as a consequence of random drift alone.

It can be expected that random extinction of mtDNA lineages in isolated populations will increase genetic differentiation among populations (Hartl and Clark 1989). Although at present only preliminary data are available, we would like to emphasize that different mtDNA haplotypes have been found in North American and European wolves belonging to different geographical populations (Wayne *et al.* 1992).

The present Italian wolf population is a viable one, particularly if the positive demographic trend currently observed will persist in the future (Ciucci and Boitani 1991). But if the effective size of this population is low, as suggested by mtDNA

analyses, then in future generations random drift will erode genetic variability also at nuclear gene loci. In order to reduce the effects of genetic drift it is necessary to favour all those measures of game management which help to increase effective population size as fast as possible.

The brown bears of the Apennines are probably isolated from those of the Alps since 400 years (E. Randi *et al.*, in prep.). Assuming $N_e/N_o = 0.25$, and a sex ratio of 1:1 (Harris and Allendorf 1989), N_e of the Apennine bear population is 20 – 25, and N_{ef} is 10 – 12. The predicted time for the mtDNA to become monomorphic is therefore $4\ N_{ef} = 40 – 48$ generations, corresponding to 400 – 480 years (generation time is estimated to be about 10 years – Harris and Allendorf 1989). Also in this case the observed mtDNA monomorphism can be explained as a result of the extinction of maternal lineages due to random drift since isolation.

The Alpine brown bears are probably less than 10 animals, so they have no chance to harbour much genetic variation. It is interesting to note that the mtDNA haplotype found in the Alps is shared with some brown bears belonging to the Croatian population (Gorski Kotar region, E. Randi *et al.*, in prep.).

By comparing our data with those of Taberlet and Bouvet (1992), and Hoss *et al.* (1992), we computed dendrograms indicating the existence of phylogeographic patterns among the West European brown bear populations studied (E. Randi *et al.*, in prep.). The subdivision of mtDNA lineages following the decline and isolation of populations was not random, but reflects a preexisting genetic divergence among populations, probably due to isolation by distance and local limitations of gene flow. According to this pattern it was possible to identify a major genetic gap separating eastern brown bear populations (Romania, Russia) from Balkan and western populations (Italy, France, Spain). The western brown bear populations are divided into three main phylogeographic assemblages: a northern lineage (Sweden), a south-eastern lineage (Croatia, Alps, Apennines), and a western lineage (Pyrenees and Cantabrian mountains). These phylogeographic assemblages must be managed separately in order to preserve the paticular genetic integrity of each of those populations. Within the south-eastern lineage, the population of the Apennines should be managed separately, because it has retained a unique mtDNA genome (which could be correlated with other unique genetic traits).

In conclusion, mtDNA sequences indicate that fragmentation and isolation of west European wolf and brown bear populations led to the fixation of different genotypes in different populations. These different mtDNA genomes indicate that genetic variability is conserved mainly between and not within populations. Where it will be possible to preserve the integrity of local genomes, we will preserve genotypes adapted to local habitat conditions. Local genomes represent the survivors of random processes working on the basis of preexisting genetic divergence due to both isolation by distance and natural selection. Thus, the analysis of genetic diversity and the individuation of phylogeographic assemblages will be of

great value in the planning of conservation and management strategies for endangered species.

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