

# PHYLOGEOGRAPHY OF BROWN BEARS IN EUROPE AND EXCREMENTAL PCR—THE NEW TOOL IN THE GENETIC ANALYSIS OF ANIMALS IN THE WILD

MICHAEL H. KOHN, Department of Biology, University of California, Los Angeles, Los Angeles, CA 90095-1606, USA, email: michaelk@biology.lifesci.ucla.edu  
FELIX KNAUER, Munich Wildlife Society, Linderhof 2, D-82488 Ettal, Germany

**Abstract:** Brown bears (*Ursus arctos*) in western and eastern Europe reportedly have different evolutionary histories. This finding is based on the occurrence of 2 distinct clades of mitochondrial DNA (mtDNA) genotypes in bear populations throughout Europe. Contact zones between populations of the 2 clades are found in Sweden and Romania. Patterns of variation below the species level raise the issue of what the unit of management should be. To investigate the nature of the contact zone in Romania, which contains both clades of mtDNA genotypes, we analyzed the spatial distribution of genotypes and the sex of the animals. One site in Romania contained individuals of both sexes that belong to either clade, thus excluding a spatial separation of individuals of different sexes from different clades. The maintenance of the contact zone is attributed to little female dispersal. Genetic data from small and endangered populations can be obtained non-invasively through the amplification from DNA sequences from excrement samples using the polymerase chain reaction (excremental PCR). Excremental PCR has provided important data on demography, genetic variability, phylogeny, and even feeding habits of the dwindling brown bear population of the Brenta Mountains in northern Italy's Trentino Province. These bears are members of the western clade and contain the same genotype as bears found in Slovenia, Bosnia, Croatia, and Romania. Restocking of the Brenta population is planned with bears from Slovenia. We analyzed genetic data of the European brown bear in terms of phylogeography and gene flow to provide a basis for management decisions.

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The use of genetic techniques to identify phylogeographic structuring below the species level was introduced by Avise (Avise et al. 1987, Avise and Ball 1990, Avise 1992). Genotypes can be phylogenetically ordered within a species, and such intraspecific phylogenies can be compared with the geographical distribution of genotypes. In fact, the genotypes can be replaced with the locations where they were found, resulting in a phylogeny of geographic distributions. This approach allows the study of patterns and processes underlying the geographic distribution of genotypes. One lesson from phylogeographics is that populations of the same species may show substantial substructuring, which is not always obvious given morphology or current taxonomic information. The importance of such phylogeographic data for the recognition of management units was stressed by Dizon et al. (1992). A closer interaction between systematists and conservation biologists was suggested by Rojas (1992). The study of phylogeographics led to the insight that populations and meta-populations deserve the attention of conservation biologists, in addition to the species as a whole (Rojas 1992).

Recently Taberlet and Bouvet (1994) and Kohn et al. (1995) reported on the phylogenetic relationships of mitochondrial control region sequences of brown bears (*Ursus arctos*) in Europe. It was shown that 2 distinct clades of mtDNA genotypes exist in Europe (Kohn et al. 1995). One clade occurs predominantly in the western populations of Europe, and Taberlet and Bouvet (1994)

suggested a further split of the western clade. The other clade is found in Eastern European populations. The Romanian population was unique in that genotypes of both clades are present (Kohn et al. 1995). We will address the importance of the phylogeographic data based on mtDNA to the management of populations of the European brown bear.

Analysis of mtDNA can produce measures of genetic variability and phylogeny that are of considerable practical relevance for conservation (Moritz 1994). Since mtDNA is inherited from the maternal line, it can only tell the matrilineal history of populations. Thus, studies of mtDNA are of special value in conjunction with assays of nuclear variation (Moritz 1994), which is biparentally inherited.

The phylogeography of bears in Europe implies that the brown bears of the western and eastern populations have different evolutionary histories probably caused by geographical separation during the quaternary ice ages. Phylogeographic splits are also found in other taxa in Europe such as field voles (*Microtus agrestis*; Jaarola and Tegelstrom 1995), grasshoppers (*Chorthippus parallelus*; Cooper et al. 1995), and oak trees (*Quercus* spp; Ferris et al. 1993). The presence of 2 distinct clades of mitochondrial genotypes of bears in Romania offers a unique opportunity to study how separation of clades is maintained and the biological significance of such a phylogeographic split. Herein, we discuss 2 mechanisms responsible for the maintenance of the contact zone.

Sampling of free-ranging animals for genetic analysis formerly required handling of the animals. However, since the introduction of the polymerase chain reaction (PCR) it is possible to obtain genetic information from minute amounts of sample. Hairs have been reported as a source for non-invasive genetic analysis of free-ranging bears (Taberlet et al. 1993). However, excrement samples are more easily found and yield cells from the intestinal lining that allow amplification of mitochondrial and nuclear sequences (Hoss et al. 1992, Constable et al. 1995, Gerloff et al. 1995, Kohn et al. 1995). This method will revolutionize non-invasive sampling of wild animals especially in small and endangered populations where animals should not be disturbed or are difficult to handle. We review the latest advances in excremental PCR and their possible contribution to the study of free-ranging animals.

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## METHODS

### Sequence Data of Mitochondrial Genotypes

As described in Taberlet and Bouvet (1994) and Kohn et al. (1995), sequence data from 128 brown bears sampled from all main populations in Europe were obtained. We used the PCR and direct sequencing of amplification products to study sequence variation of the mitochondrial control region (Kohn et al. 1995). The primers employed for amplification and sequencing are described in Taberlet and Bouvet (1994) and Kohn et al. (1995). For all individuals sampled, 213–218 base pairs (bp) were aligned and compared. Genotypes were assigned to sequences with unique nucleotide substitutions. We computed a 50% majority-rule consensus tree (Kohn et al. 1995), and the support for nodes was estimated by 1,000 replicates of bootstrap analysis (Felsenstein 1985). We compiled phylogeography by adding geographic distribution (sampling locations) of genotypes to the phylogenetic tree.

To gather demographic information of the Brenta population, we amplified a short segment of the nuclear Y chromosomal *SRY* gene, a sex determining factor. The primers for amplification and sequencing are described in Taberlet et al. (1993).

### Samples from Romania

To study the nature of the contact zone of the western and eastern clade in Romania we analyzed 26 individuals of the Romanian population from 5 locations. Samples from location 1 (Bistritia) included 9 males, 6 females, and 5 individuals of unknown sex. At location 2 (Tirgut Ocna), 1 male was sampled. At location 3 (East Brasov), 3 males were found, and at location 4 (Sinaia) and location 5 (West Brasov), 1 male was sampled. Samples came from legal hunting in Romania. The sex of the animals was provided in the hunting records.

### Extraction

DNA was extracted from tissue using phenol-chloroform. We also successfully amplified DNA from liver samples without any extraction step by inoculating the PCR reaction with a pipette tip that had been pierced into the liver sample (Kohn et al. 1995).

It was impossible and undesirable to obtain tissue or blood samples from the bears of the Brenta, thus we developed a novel, non-invasive approach to study genetics of wild animals: excremental PCR. Excrements were sampled and stored dry at 4 C or in ethanol. In contrast to a phenol-chloroform procedure, this technique has the advantage that substances which inhibit the PCR reaction can be removed from the extract. The technique is a silica-based extraction procedure (Hoss et al. 1992) first employed by Boom et al. (1990). About 50 mg of dry excrement were added to the extraction buffer L6, suspended, and transferred to a mixture of L6 and the DNA-adsorbing silica beads (Hoss et al. 1992). Three washing steps and elution of the DNA from the silica beads were necessary before the PCR reaction was initiated. The protocol is described in detail in Kohn et al. (1995).

## RESULTS

### Phylogeography of the European Brown Bear

Kohn et al. (1995) identified 20 genotypes, Taberlet and Bouvet (1994) identified 16 genotypes, and 8 genotypes among the 2 groups were found to be identical,

resulting in a total 28 different genotypes. A 1–6 bp insertion–deletion was omitted from the analysis, leaving 17 lineages for phylogenetic relationships analysis using PAUP 3.1.1. (Phylogenetic analysis using parsimony, Swofford 1993).

Two distinct clades of related mitochondrial genotypes were found in Europe (Fig. 1). Mean pairwise sequence differences within the western clade are between 0.0–6.2 nucleotide substitutions, and between 0.0–0.3 nucleotide substitutions within the eastern clade. Mean pairwise sequence divergence between the 2 clades ranges from 14.1–16.1 base substitutions. The geographic distribution of the 2 clades is summarized in Figure 2.

The relationship between populations is also reflected in the presence or absence of shared genotypes. Shared genotypes indicate common ancestry of populations or past or present gene flow between populations. Populations with single, unique genotypes are found in the French Pyrenees, Greece, Norway, and southern Sweden (Taberlet and Bouvet 1992, 1994). The populations in the Brenta, Abruzzia, Bosnia, Bulgaria, Finland, and northern Sweden appear to be monomorphic for 1 genotype, but the genotype is shared with other populations. The genotype found in the Brenta is also found

in Bosnia, Croatia, Slovenia, and Romania. The genotype found in Abruzzia is also present in Slovenia. The genotype found in Bulgaria is shared with the Romanian population. One of the 4 genotypes found in Slovakia is also present in Russia. In the populations of Finland, northern Sweden, Russia, and Estonia a common genotype was found in all samples. Russia also shares a genotype with Romania.

The within–mean pairwise sequence divergence of the Romanian population is highest (7.9,  $n = 27$ ), followed by the population from Bosnia, Croatia, and Slovenia (0.5,  $n = 34$ ), Estonia, Finland, northern Sweden and Russia (0.3,  $n = 23$ ). The population of Slovakia exhibits variation only in a 1–6 bp insertion–deletion.

### The Contact Zone in Romania

The Romanian population can be viewed as a continuous population with one of the highest densities in the world (W. Schroder, Munich Wildlife Society, Ettal, Germany, pers. commun., 1992), as the management for the past few decades has been oriented toward trophy hunting. To investigate whether there is a spatial separation of members of the 2 clades, the sex and the genotype of 26 individuals were compared. At location 1 we found that 7 males, 4 females, and 1 individual of unknown sex had genotypes of the western clade. At the same location 2 males, 2 females, and 4 individuals of unknown sex showed genotypes of the eastern clade. At location 2, 1 male of each clade was sampled. At location 3, 1 male of the eastern clade and 2 males of the western clade were sampled. At locations 4 and 5, 1 male each of the western clade as sampled.

### Excremental PCR

Preliminary experiments showed that mtDNA can be successfully retrieved from feces of elephant (*Elephas maximus*), European bison (*Bison bonasus*), polar bear (*Ursus maritimus*), and brown bear (Kohn et al. 1995). Those feces were sampled from zoo animals and immediately processed. Samples from the wild brown bear population were collected over the course of 3 years. We were able to amplify DNA from 8 out of 12 fecal samples. The length of the products that could be amplified were 398 bp, 295 bp, or 141 bp. We also used hair samples found in the field to study the genetics of the bears from the Brenta. Of 16 samples, 15 resulted in a 398 bp amplification product.

To obtain demographic information of the Brenta population, we amplified 134 bp of the Y-chromosomal

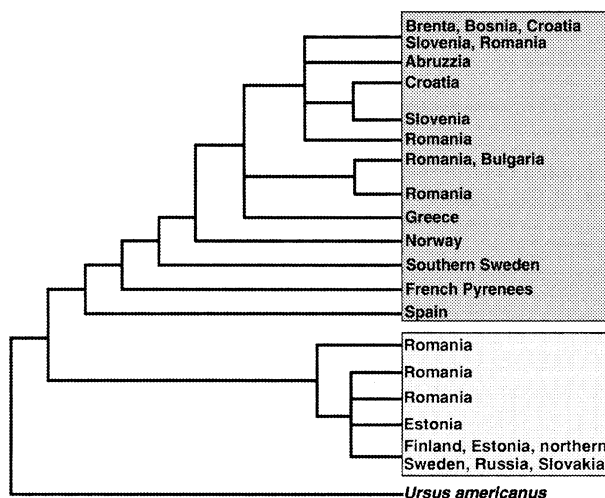


Fig. 1. Maximum parsimony phylogenetic tree (50% majority-rule consensus) of 28 mitochondrial genotypes identified in the European brown bear using the data from Taberlet and Bouvet (1994) and Kohn et al. (1995). Six equally parsimonious trees were found that explained the deep dichotomy in the genepool of the European brown bear. Bootstrap analysis supported no other node than the split between western genotypes (dark grey) and the eastern genotypes (light gray). The American black bear was used to root the tree.

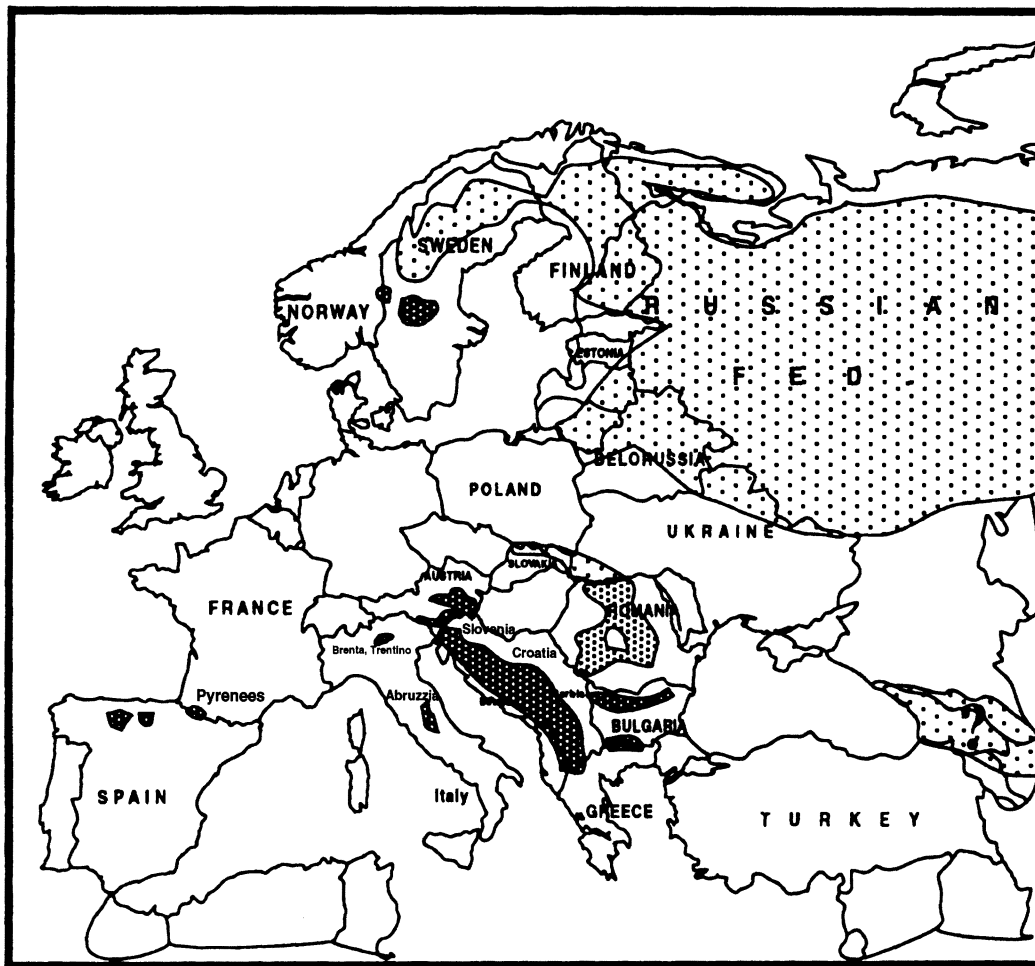


Fig. 2. Geographic distribution of genotypes identified by mitochondrial control region sequences of 128 European brown bears (Taberlet and Bouvet 1994, Kohn et al. 1995). Genotypes of the western clades (dark pattern) are found in the populations of Spain, the French Pyrenees, southern Sweden, Norway, Greece, Slovenia, Croatia, Bosnia, Italy, Austria, and Romania. Mitochondrial genotypes of the eastern clade (light pattern) were found in Finland, Estonia, northern Sweden, Russia, Slovakia, and Romania. The Romanian population (intermediate pattern) contains genotypes from both clades.

*SRY* sequence from 4 excrements and 4 single hairs. Of those 8 samples, 4 were of male origin and 4 of female origin.

## DISCUSSION

### Phylogeography Is a Result of Evolutionary Events

The European brown bear exhibits substantial genetic variability. The gene pool of the bears in Europe is divided into 2 clades of related mitochondrial genotypes (Fig. 1). Populations of 1 clade have a western distribution in Europe; populations of the other clade have an eastern distribution (Fig. 2) with contact zones

in Sweden (Taberlet et al. 1995) and Romania. Such phylogeographic splits were described also for other taxa in Europe (Ferris et al. 1993, Cooper et al. 1995, Jaarola and Tegelstrom 1995).

Although bear populations have drastically declined in all of the western populations and some of the eastern populations due to overhunting and habitat loss, this cannot explain the dichotomy in the gene pool of the European brown bear. Random extinction of genotypes due to human activity would not result in a congruent pattern of phylogenetic relationships of genotypes and their geographic distributions. These clades can be best explained by geographic separation of brown bear populations by icesheets or unsuitable habitat during the quaternary ice ages. Thus, the ob-

served pattern results from the evolutionary history of brown bears in Europe.

No evidence for a phylogeographic split of the European brown bear was suggested by morphology or behavior. This illustrates the power of molecular techniques to retrace evolutionary histories not only for species, but also below the species level. However, mitochondrial DNA is inherited in a maternal fashion. Thus, the observed pattern is shaped by female dispersal. Nuclear markers that are biparentally inherited have yet to demonstrate that the phylogeographic split is also found in the nuclear genepool of the European brown bear. Whereas mitochondrial DNA is a neutral marker, nuclear markers may be linked to genomic regions that could be affected by differential selection (Moritz 1994).

Detection of patterns of gene flow by shared mtDNA genotypes is a conservative approach. However, a former population ranging from Abruzzia, Brenta, Slovenia, Bosnia, and Croatia with contact to Romania can be reconstructed when populations with shared genotypes are connected. Patterns of gene flow are better investigated when large samples of adjacent populations are available and nuclear markers are studied. Furthermore, populations presently monomorphic for a single mitochondrial genotype may have been more diverse in the past. An assessment of the gene pool of monomorphic populations before their decimation would be important; skin samples from the beginning of the century may serve as the source of genetic material (Thomas et al. 1990). Our results are intended to stimulate studies of the present nuclear variation, as well as studies of past mtDNA diversity in the European brown bear.

### The Contact Zone in Romania

The occurrence of a contact zone between the 2 clades of related genotypes in Romania poses the question of how such a contact zone is structured and maintained. At location 1, the Bistritia area in northeastern Romania, bears from both clades and both sexes are found on a microspatial scale side by side. This excludes a mechanism that leads to spatial separation of members of different clades. Since mitochondrial DNA is inherited maternally, the contact zone could result from limited female dispersal. Dispersal data using radiotelemetry are necessary to test this hypothesis (see also Taberlet et al. 1995), but bears dispersing from Slovenia into the Austrian Alps are almost exclusively male individuals (W. Schroder, Munich Wildl. Soc., Ettal, Germany, pers. commun.). The contact zone we observe today was probably established by females of both clades that recolonized central Europe from Asian and western European

refugia. Nuclear markers have yet to confirm that males have not already dispersed further and interbred with females of the opposite clade. However, geographic isolation over substantial time may lead to fixation of genetic and behavioral traits. This could lead to a situation where bears from the 2 clades preferentially mate with bears of the same clade. In fact, such a situation was found in pocket gophers (*Thomomys* spp., Patton and Smith 1993) and grasshoppers (*Chorthippus* spp., Butlin and Ritchie 1991). Further, differential fitness of offspring between members of different clades may be detected. Contact zones offer a unique opportunity to study species or subspecies boundaries (e.g. Harrison 1990).

Research concerning distributions of nuclear markers such as microsatellites needs to be conducted in the Romanian population. If Hardy-Weinberg disequilibrium were detected, assortative mating or differential fitness of offspring would be legitimate explanations.

Locations 2 through 5 do not provide insight into the nature of the contact zone. Only males were sampled, which is attributed to the bias in hunting toward males. We suggest that additional unbiased sampling is necessary to obtain a picture of the contact zone.

During the cold war, an unknown number of zoo bears with unknown origin were released in Romania. Thus, the composite gene pool of the Romanian population may be an artificial phenomenon. However, contamination of the Romanian population with western genotypes seems to be unlikely because bears released in Romania were certainly more easily obtained from eastern European countries than western European countries.

### Excremental PCR

This method was specifically developed to obtain genetic data from a tiny population of brown bears in Brenta, northern Italy (Fig. 2). One mtDNA genotype was found in the area (Kohn et al. 1995), and the closest related population is from Slovenia, Croatia, and Bosnia.

In the Brenta region, excrement samples were scattered over a large area and were more easily found in the field than hair samples (A. Stoffella, Stazione Forestale Andalo, Andalo, Italy, pers. commun., 1992). Since this was the first time that we used excrement samples to study the genetics of a wild population, we also sampled hair to ensure the accuracy of our data.

In our study, the DNA yield appears to be lower when compared to hair. This is reflected in the higher number of successful amplifications from hair as well as in the higher quality of the DNA retrieved from hair (Kohn et al. 1995). However, since we were able to obtain single copy nuclear DNA from fecal samples, the quality of the

DNA in fecal samples is also suitable for studies of nuclear genetic variation (Constable et al. 1995, Gerloff et al. 1995, Kohn et al. 1995). Four samples each from excrement and hair were used for sex identification; 2 from each source yielded a 134 bp Y-chromosomal *SRY* segment and therefore stemmed from male bears. The other 4 samples stemmed from female bears (Kohn et al. 1995).

Excremental PCR can provide many different kinds of information in a completely non-invasive fashion on wild animal populations. Food habits can be studied by genetic analysis (Hoss et al. 1992). Infection of animals with parasites such as viruses, bacteria, and other macroparasites such as tapeworms can be detected (Frankel et al. 1989, Sidransky et al. 1992, Bretagne et al. 1993). In addition, the sex ratio of a population can be studied by amplifying the nuclear Y-chromosomal sequence *SRY* (Kohn et al. 1995); thus estimates of effective population size can be obtained non-invasively. Moreover, individuals can be identified and parentage can be determined; thus the reproductive success of individuals can be studied when microsatellites, highly variable nuclear markers, are amplified (Constable et al. 1995, Gerloff et al. 1996).

Excremental PCR is an especially valuable tool for non-invasive genotyping when animals are elusive or invasive sampling is undesirable. For example, handling of animals to take blood samples or to attach a radiocollar is viewed as disrespectful toward the animal in some cultures and can negatively influence the relationship between scientists and native people in the study area. When behavior studies are combined with the genetic analysis of animals, it is also undesirable to handle the animals because handling may alter their behavior. We expect excremental PCR to be applied in the future for purposes such as counting animals in the wild. This may replace expensive and time-consuming methods such as capture-recapture estimates of population sizes.

In our opinion it is necessary to draw attention to the fact that excremental PCR, as other PCR-based methods is prone to contamination (carry-over of non-target template DNA). This is especially true when tissue samples and excrement samples from the same or closely related species are handled in the same laboratory. To monitor for contamination, we strongly encourage the use of negative controls, mock extractions, and mock PCR amplifications alongside with the extraction and amplification. Further, we strongly recommend a spatial separation of the extraction, the setup of the PCR, and the processing of amplification products. In addition, excrements may contain DNA from food that animals have consumed as well as bacterial DNA (Hoss et al. 1992, Kohn et al. 1995).

Thus, amplification products obtained from excrement samples should be sequenced to verify the origin of the DNA. This is especially important for studies using microsatellites where primers are used that are conserved over a range of taxa. It seems that the retrieval of authentic sequences from carnivores is more difficult than from herbivores; this was obvious when we failed to obtain mtDNA information from zoo tigers (*Panthera tigris*). We found it useful to test the primers applied in our study on DNA of human, roe-deer (*Capreolus capreolus*), horse (*Equus caballus*), and fox (*Vulpes vulpes*) to test the likelihood of amplifying other taxa present in the study area. Testing of closely related taxa is even more critical in areas where black bears (*Ursus americanus*) and brown bears are sympatric.

## MANAGEMENT IMPLICATIONS

### Restocking and Augmentation

Eastern and western populations of the European brown bear can be distinguished by mitochondrial control region sequences. This is the result of the evolutionary history of the brown bear in Europe, and the clades should be preserved or reestablished. If restocking and augmentation projects aim to restore the original gene pool, bears from eastern populations should not be used to restock the endangered western populations. Populations of the 2 clades should be viewed as different management units until information of nuclear markers is available. The population from Slovenia, Croatia, and Bosnia is the most suitable population to restock or reintroduce western populations, especially in the Alps. First, it is closely related to the other western populations; second, bears from this population have started to recolonize old former bear habitat in the Austrian and Italian Alps; and third, this population exhibits the highest amount of genetic variability within the western clade. How this population is affected by the war in former Yugoslavia has to be evaluated before individuals are removed. The Brenta population, although almost extinct, is an important stepping-stone population for the reintroduction of brown bears in their former range in the Alps.

### Forensics

Several small populations are monomorphic for 1 mitochondrial genotype, thus individuals or remains stemming from these areas can be identified. However, populations with unique genotypes may result from sampling artifacts in the large populations, which may contain those genotypes at low frequencies. For example,

the Romanian population contains the genotype from the Brenta at a frequency of 4.4%; in fact, the genotype was found once among 25 individuals. Sampling is very critical in this context. As another example, Taberlet and Bouvet (1994) concluded that the Romanian population belongs to the eastern population based on 2 samples that had eastern genotypes.

## Excremental PCR

Besides the applications suggested above, 1 tempting application is the identification of individual bears involved in bear-human conflicts. For example, identification of animals responsible for the killing of livestock can be identified by excrements found in the vicinity of the kill. Since excrement samples are readily found in the field, we suggest excremental PCR as a tool for monitoring wild animal populations. This may be especially useful in small and endangered populations, where the sex-ratio and inbreeding are of concern.

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