UNIVERSITÀ DI PARMA

Dipartimento SCVSA

Laurea Magistrale

in Ecologia ed Etologia per la Conservazione della Natura

Investigating changes in neutral genetic variability of the brown bear (*Ursus arctos*) population of Trentino, Italy in the two decades following its reintroduction

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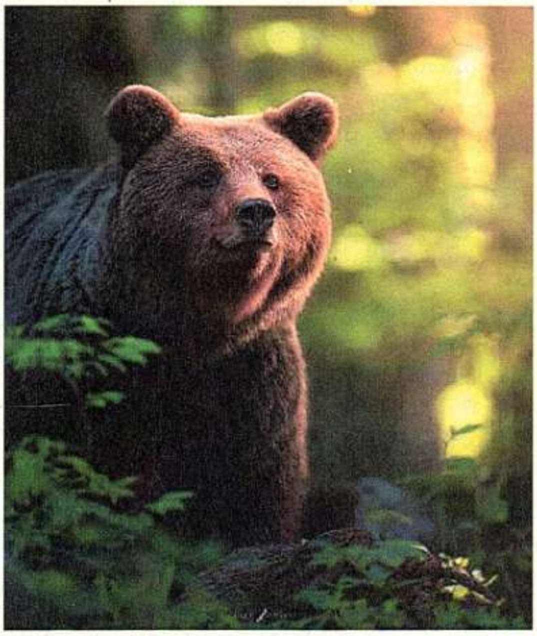
Anno accademico 2020/2021



The brown bear is a symbol of wilderness and our desire for freedom. But, above all, with the return to the forests from which we drove him away, it helps us to reflect on how we want our relationship with nature to be

1. INTRODUCTION

The importance of preserving biodiversity as a whole is now well-recognized, as numerous studies have shown that species richness and high levels of genetic diversity increase the productivity and stability of ecosystems and their resistance to invasion of alien species, as well as reduce the rate of transmission of diseases between species within ecosystems. In addition, high levels of genetic variability increase the capacity of species to adapt to changes in their environment (Worm *et al.*, 2006; Tilman, 2012; Soulè 2013).

The environmental and cultural importance of the bear is reflected in the regulations, that grant the species one of the highest degrees of protection at the European level (Zibordi, 2017).

In Italy, in particular, in order to save the small nucleus of brown bear (Ursus arctos) remaining on the Brenta Mountains from certain extinction, at the end of 1990s the Adamello Brenta Natural Park initiated the Life *Ursus* Project financed by the European Union, which aimed to release a small number of wild Slovenian bears in Trentino (Davoli *et al.*, 2015). The final objective of the project was the restoration of a viable bear population in the central Alpsa goal recently achieved when it was estimated that the numerical consistency of about 80 brown bears had been reached. (Groff et al., 2019). But several questions remains; for example, what about their genetic variability? Can we say the presence of this species will be viable in the long term?

1.1 NON-INVASIVE GENETIC MONITORING

This type of monitoring is defined as 'genetic' because it is based on the analysis of molecular markers, and 'non invasive' because the DNA is from biological samples collected in the environment (usually hair and feces), thus avoiding directly manipulating the animal (Davoli *et al.,* 2013). The collection of the samples can be carried out through both systematic sampling (following a predefined grid, with the use of *hair-traps,* for a limited period of time) or opportunistic sampling (that is the collection of samples during the regular activities of the park rangers throughout the year). Non-invasive sample collection is advantageous for genetic studies of large carnivore populations, since traditional observational. Monitoring of the bear, as well as other large carnivores, presents operational difficulties, related to the elusiveness of the species, nocturnal habits, very low densities, long dispersal distances, and prolonged period of winter inactivity (AA. VV., 2010b). Therefore, non-invasive genetic monitoring is one of the primary ways of acquiring information about brown bears in the central Alps (Fattori *et al.,* 2010).
DNA is a delicate molecule, which degrades easily under conditions of high humidity, high temperature or temperature oscillations, direct sunlight and interaction with chemicals. One of the difficulties of working with non-invasive

biological samples is related to their high rate of DNA degradation (De Barba *et al.*, 2017). Contamination of the sample from other DNA sources, such as soil and the field technicians themselves, can also compromise genetic analyses. Thus, appropriate sample collection, conservation and storage is necessary to minimize these problems (Taberlet *et al.*, 1999; Waits *et al.*, 2005; AA. VV., 2010b).
The molecular marker generally used for the identification of a species is a region of mitochondrial DNA with interspecific variability, such as differences in length or sequence composition. Instead, individual identification is carried out through the typing of a number of molecular markers, called microsatellite loci, necessary and sufficient to distinguish one bear from another, as if it were a genetic fingerprint (DNA fingerprinting). Microsatellite (or "STR" – *short tandem repeats*) are regions of non-coding repetitive DNA units consisting of very short repeat units (1 to 5 pairs of bases) scattered throughout nuclear DNA. Microsatellites have a high level of polymorphism, due to the different length of the sequence between individuals in a population, including between parents and offspring and between siblings and, therefore, are very informative markers in population genetics studies. The length variants at any one locus are called 'alleles'; as long as the set of loci have a high enough level of polymorphism within and across populations, the analysis and identification of alleles allows an individual's multilocus genotype, which is the set of alleles carried by an individual for the loci taken into consideration, to be distinguished from that of other bears (Fattori *et al.*, 2010; Davoli *et al.,* 2013). Using population genetic analysis, the same dataset can be used to generate valuable information on demography (population size, reproduction, mortality), ecology (distribution, habitat use) and genetic variability. For wild populations, the growing development and application of molecular markers obtained from samples collected non-invasively, provide new possibilities for establishing kinship and reconstructing pedigrees even in species where such information cannot be obtained from field observations alone, and, above all, that have a high level of elusiveness (as in the case of the bear). This information is crucial for following the trend of the population over time to ensure appropriate management for the conservation of the species (AA. VV., 2010b; Davoli *et al.,* 2013). Long-term monitoring is particularly important following reintroductions or translocations for assessing the success of such programmes and for ensuringe prompt actions for improving the status and probability of population persistence (Miller *et al.*, 1999). In addition, reintroduction programmes often release only a small number of individuals; therefore, effective population sizes are initially small and monitoring changes in genetic parameters is of primary concern for population viability (Frankham 2005; see text for details). In the absence of gene flow, levels of coancestry are expected to rise; genetic diversity is lost as a result of drift and mating between relatives; adaptive evolutionary potential is limited, and accumulation of deleterious alleles is accelerated (Ralls *et al.* 1988; Hedrick 2000).

1.2 STUDY SPECIES – Brown bear (*Ursus arctos*)

1.2.1 SYSTEMATICS AND DISTRIBUTION

All extant bear species belong to the Family *Ursidae* (Class Mammalia; Order Carnivora) (Table 1). Together with *Canids*, they are grouped in the suborder Caniformia (Flynn *et al.*, 2005). This Family consists of 5 genera that include the largest terrestrial predators distributed throughout the temperate zones of the northern hemisphere; only a few species have an equatorial distribution (Teofili C., 2006). Asia hosts four out of five genera, one of which is also found in Europe, North America and the Arctic Circle: genus *Ursus*. The fifth genus, *Tremarctos*, is only found in South America (Wilson & Mittermeier, 2009).

Table 1 Systematic classification of brown bear (*Ursus arctos*)
(modified from *www.itis.gov*)

Class	Mammalia (Linnaeus, 1758)
Order	Carnivora (Bowdich, 1821)
Suborder	Caniformia (Kretzoi 1938)
Family	Ursidae (Fischer de Waldheim, 1817)
Genus	Ursus (Linnaeus, 1758)
Species	*Ursus arctos* (Linnaeus, 1758)
Italian subspecies	*Ursus arctos arctos* (Linnaeus, 1758)

The brown bear (*U. arctos*), is a large mammal described by Linneus in 1758, one of 8 species of the Family *Ursidae*. Currently, 7 other species also belong to this Family: the Giant panda (*Ailuropoda melanoleuca*), the Spectactled bear (*Tremarctos ornatus*), the Sun bear (*Helarctos malayanus*), the Sloth bear (*Melursus ursinus*), the Black bear (*U. americanus*, Pallas, 1780), the Polar bear (*U. maritimus*, Phillips, 1774) and the Asian black bear (*U. tibetanus*, Cuvier, 1823)(Fig. 1).

Fig. 1 Worldwide distribution of the species belonging to Family *Ursidae* (www.parcoabruzzo.it)

In particular, the brown bear in Trentino (*U. arctos arctos*) is considered to belong to the same subspecies as that found in Croatia, according to genetic studies on mitochondrial DNA. Based on this, it is possible to consider the Trentino nucleus as a residual of an original population that occupied the entire area from the Alps to the Balkans.

In the past, some authors proposed a particularly complex classification for *U. arctos*, dividing the taxon into numerous different species and subspecies, probably due to the lack of data on large portions of its range, as well as to its remarkable phenotypic variability (Mustoni, 2004). Over time, however, the taxonomy was simplified, leading to the currently accepted presence of a single species of brown bear (Couturier, 1954). This species has the widest distribution of all the bears, and is abundant across Europe, Asia and north-western America from arctic tundra to the subtropical regions (Kopatz A., 2014; Zibordi, 2017; Fig. 2). Hence, this species is considered one of the most successful mammals in this climatic zone, thanks to a capacity to adapt to a range of different environments.

Fig. 2. Worldwide distribution of brown bear (dark grey). The map is freely available at Wikimedia commons. The original colours were converted to greyscale

Although the brown bear is still abundant in northern and eastern Europe, in the southern part of the continent the situation is undoubtedly critical: the populations present are few, isolated and often characterized by conflicts with humans that pose serious doubts about their future (AA. VV., 2010a). Most populations have already reached a critical level of extinction risk. This trend is the result of habitat loss and direct persecution by humans that have in turn resulted in small population size, genetic isolation (no or low rate of mating of individuals between populations) and low genetic variability, negative factors that contribute to inbreeding depression. The latter is defined as the probability that some individuals have identical alleles at some loci by hereditary transmission from a common ancestor. It often occurs in small populations since mating between related individuals is highly likely, rather than random mating. Inbreeding typically increases the proportion of homozygotes, allowing recessive lethal alleles to express themselves in the phenotype. For this reason, maintaining natural levels of heterozygousity within populations is of particular importance. It has been widely shown in numerous studies that inbred populations have a lower rates of survival to sexual maturity, as they are more likely to have genetic diseases from recessive alleles, a lower immune response, and therefore higher mortality rates, especially with regards to juveniles.

This can lead to population decline in following generations (O'Grady *et al.*, 2006; Walling *et al.*, 2011; Dunn *et al.,* 2011; Relethford, 2013; Wang *et al.*, 2020). Thus, a measure of genetic diversity within populations allows to obtain an indirect estimation of both their effective size and the trend of inbreeding (Mustoni, 2004).

Fig. 3. Brown bear distribution in Italy (www.parcoabruzzo.it)

In Italy, the brown bear survives in two geographically isolated populations: a small relict of the original pan-Alpine population in Trentino (*U. a. arctos*), with about 80 individuals; the other *U. a. marsicanus*, in the central Apennines, where about 50-100 individuals occupy a continuous range from the Abruzzo, Lazio and Molise National Park in the sout, to the Majella mountain massif in the north, with sporadic reports on the Reatini, Duchessa, Gran Sasso, Laga and Sibillini mountains (Davoli *et al.,* 2015; Fig. 3). Moreover, about ten individuals are also present in the Friuli-Venezia Giulia Region, but thanks to genetic analysis these are all males dispersing from Trentino and Slovenian populations; in fact, they all have the same mitochondrial DNA haplotype and are recognized individually by their microsatellites (STR) genotype, that allow researchers to define which bears they are and where they come from (Fattori *et al.*, 2010; Filacorda *et al.*, 2017).

1.2.2 MORPHOLOGY AND PHYSIOLOGY

The brown bear is a robust mammal, characterized by a heavy build and dark brown coat, often with reddish reflections and black, gray and beige shades. There is a great variability in pelage colour, from very dark brown to beige, and almost white (Filacorda *et al.*, 2017; Zibordi F., 2017).

Fig. 4. Brown bear with cubs (Ph: Miha Krofel, from www.dinalpbear.eu).

Coat colour does not depend on sex, geographical location or genetic background; however, older individuals tend to have fur that is less thick and shiny, with bleached hairs, and cubs have a white collar that generally disappears in adulthood (Fig. 4).

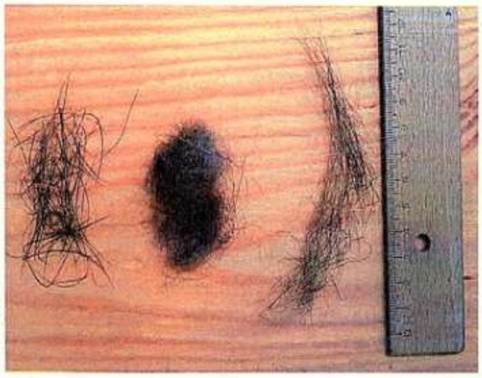
Fur is composed of three types of hair (Fig. 5):

- Giarra hair: constitutes the outer layer of the fur; these hairs are stiff and vary in length from 5 to 15 cm; they are distributed evenly over the body and perform a protective function (Daldoss, 1981; Teofili, 2006).

- Wad hair: are in direct contact with the epidermis; these are shorter (1 to 5 cm), softer and twisted to form a thick fluff under the guard hairs (Couturier, 1954; Daldoss, 1981; Mustoni, 2004). Wad hair is distributed in the areas of the body more exposed to cold (e.g. on the back) and represent an effective barrier against heat loss.

- Paw hair: similar to guard hairs, but shorter and more rigid, growing under the paws, between the digits and the pads of the foot (Teofili, 2006).

Fig. 5. Brown bear hair. From the left: silky hairs of the underpaws, wad hairs and giarra hairs (Mustoni, 2004).



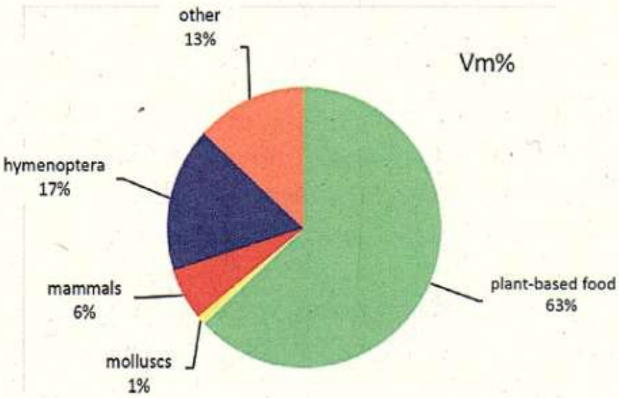
The size of brown bears varies greatly between individuals, sexes, age groups and seasons. In general females are smaller than males; for example, in the Alps the mean weight is around 150 kg for adult males (although they may exceed 300 kg); while females weigh between 70 and 160 kg, with a mean of 90 kg. This species continues to grow from birth to more than 10 years of age (Mustoni, 2004; Filacorda *et al.*, 2017; Zibordi, 2017).

The skull, mandible and teeth of bears are adapted to an omnivorous diet (Teofili, 2006). For example, the fourth upper premolar (P^4) and the first lower molar (M_1) are not typical Carnivore canines, and are flat rather than pointed. Moreover, their flat molars, called bunodonts, are used for shredding food of various kinds, not only for a carnivorous diet (Couturier, 1954; Daldoss, 1981; Teofili, 2006). Therefore, chewing is less effective than that of other Carnivores and bears are not able to finely shred food before swallowing (Teofili, 2006). In addition, the gastrointestinal system is not comparable, by development or digestive capacity, to that of vegetarian mammals and thus, much of the ingested plant mass is not completely digested, leading to the defecation of large, easily identifiable fecal pellets with plainly visible whole seeds and partially digested vegetable matter.

1.2.3 DIET

Although it belongs to the order *Carnivora,* the brown bear is considered omnivorous, with a strong preference for plant-based food. Numerous studies highlight its enormous capacity to adapt to local diet availability. The bear can also modify its eating habits based on short-term environmental modifications by humans (Elgmork, 1978; Zunino, 1981; Clevenger & Purroy, 1991). For this reason the brown bear is often described as an 'ecological opportunist' (Murie, 1948 in Johnson, 1982; Herrero, 1978; Fabbri, 1988; AA. VV., 2002) or as an 'omnivorous opportunist' (Grosse, 1999). As a result, it is difficult to make a complete list of dietary items it can potentially exploit.

Fig. 6. Mean composition of brown bear diet in a protected area (modified from Adamello-Brenta Natural Park 2002, www.parcoadamellobrenta.tn.it)



In a study carried out in the Province of Trento, Italy during the *Life Ursus* reintroduction project, vegetable matter was found to make up 63% of the diet (Fig. 6), while ants represent the next most consumed food category (AA. VV., 2002). In fact, brown bears balance their diet by consuming both insects and mammals (i.e. livestock animals, wild ungulates and micro-mammals, such as rodents), which provide essential amino acids (Mustoni, 2004). In spring the vegetative parts of plants are very important because of their high protein content and low percentage of lignin and cellulose, organic material that bears cannot digest. (Hamer, 1987). In this season, the carcasses of wild ungulates that have perished during the harsh winter months may also be consumed (Braña *et al.*, 1988; Clevenger & Purroy, 1991). In summer the consumption of wild fruit and nuts, insects and their larvae also become significant. In autumn, a critical period that precedes winter hibernation, bears continue to eat wild fruit and, when possible, apples and pears from orchards (Clevenger & Purroy, 1991; Frackowiak, 1992; Osti, 1999). In winter, when not hibernating, brown bears are 'food generalists', feeding on what they can find. Micromammals are also preyed on occasionally in this season. (Clevenger & Purroy, 1991). Beehives are occasionally raided by particularly bold individuals, and domestic animals are rarely killed and consumed. It should be remembered that it is energetically disadvantageous for bear to prey on living animals, so able to escape (Shwartz, 1991; Mustoni, 2004). Therefore, given its remarkable adaptability it prefers to diversify its diet and find food in other ways. This is the evolutionary path followed by the bear, becoming an inefficient predator and an opportunistic omnivorous (Clevenger & Purroy, 1991). However, although predation events on domestic livestock are rare, the period in which they occur most frequently seems to be spring, when greater amounts of protein are required (Mustoni 2004; Zeni, 2016).

1.2.4 HABITAT AND DISPERSAL

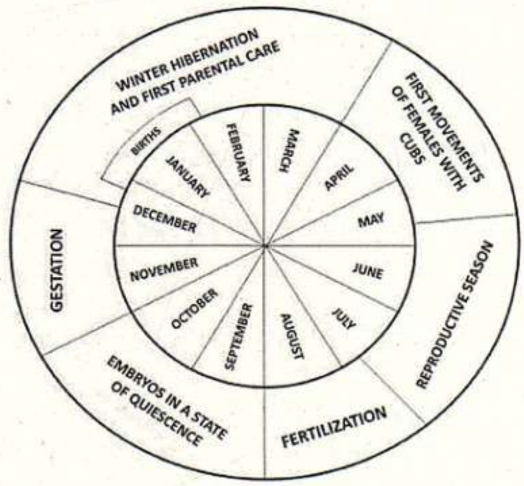
A very important ecological requirement for the brown bear is the availability of vast territories, with a high environmental diversity to provide both the necessary food resources, refuge areas and mates (Couturier, 1954; Boscagli, 1988). Although the preferred habitat of this species is deciduous forests because of their high quality and quantity of trophic sources (Ciucci & Boitani, 1997; Clevenger *et al.*, 1997), the bear is also present in very diverse environments both from a vegetation and morphological point of view. In the Alps, this species is present in forests between 300 and 1400 m a.s.l.; according to Couturier (1954) the bear is confined to these mountainous areas with rich vegetation to avoid competition with humans. However, the belief that this species prefers undisturbed areas with little human presence is false: instead, it seems to be well-adapted to different habitats and paradoxically, some may depend on anthropic food sources. So, it needs only small areas in its territory (even a few hectares) where the vegetation cover makes the probability of encounter with humans very low (Mustoni, 2004). In summary, for the vitality of a population as a whole, a rich and differentiated ecosystem is needed (Duprè *et al.*, 2000).

In order to find sufficient food resources and mates, the brown bear can move large distances that lead it to extend its *home range*. Despite this behaviour, it cannot be defined as a territorial animal, because it does not actively defend this large area probably because it would require too much energy (Lovari, 1987; Boscagli, 1988; Huber & Roth, 1993); therefore, the benefits of territoriality, such as resource reliability, are diminished in this species (Steyaert *et al.*, 2012). Several European studies on radiocollared bears have highlighted that individuals of this species walk about 2 km per day, and adult males range farther than females and subadults (AA. VV., 2000). In fact, females are generally 'philopatric', and settle close to the area where they were born and raised (Zeni, 2016), while males that separate from their mother (15-17 months of age) start to move considerably, sometimes hundreds of kilometers away from their birthplace. This behaviour is more correctly called 'exploratory displacements' as such males often return to their original area for the reproductive season, and then move away again. There is, however, a remarkable individual and seasonal variability in the extent of their movements (Mustoni, 2004). Young males born in Trentino have been recorded in neighboring European countries to the north and west (Germany, Austria, Switzerland), as well as Italian regions to the west (Lombardy), south (Veneto) and east (Friuli-Venezia Giulia) (Zeni, 2016). On the contrary, the spontaneous arrival in Trentino of males dispersing from other populations, such as that in Slovenia (the closest at ~ 300 km to the Slovenian border) has not been recorded, presumably due to the ecological barrier posed by the wide Adige valley, dominated by human presence and anthropogenic infrastructure. This one-way flow of individuals means the Trentino bear population is isolated, with potentially negative consequences on levels of genetic variability (Zeni, 2016; Corradini *et al.*, 2020).

1.2.5 LIFE CYCLE AND REPRODUCTION

The brown bear is considered to be a long-lived animal since the maximum age of life in the wild is 20-25 years for females (Teofili, 2006), and 30 years for males (Schwartz et al. 2003; Zedrosser *et al.,* 2007). Both sexes become reproductively mature at 3-4 years old, but they only begin to actively participate in reproduction about 4-5 years of age, although females may begin earlier as a result of 'environmental conditioning' (quality and quantity of food resources; Mustoni, 2004), as is generally the case in the Trentino population. Female fecundity increases with age, and for those that have multiple litters, the interbirth interval is 2 years (De Barba *et al.*, 2010). The minimum peak of fecundity in females is reached around 20 years, after which senescence begins (Schwartz *et al.,* 2003). No male older than 27 has been documented to be reproductive (Schwartz *et al.,* 2003; Zedrosser *et al.*, 2007). Generation time can change within populations; for example, in the Alps it is considered to be 4-5 years for females, and 5-6 years for males, with a mean of 5 years (Pedrotti L., *pers. comm.*)
The brown bear is generally considered a solitary species, socializing only during the reproductive season (AA.VV., 2002). In the late spring, males actively start to search out mates, moving considerable distances, probably based on scent marks left on the ground by receptive females (Daldoss, 1981; Clevenger *et al.*, 1992). Mating pairs are formed at the beginning of May (Mustoni, 2004). Copulation takes place between the end of May and the end of July, with a peak in late June and early July (Couturier, 1954; Daldoss, 1981; Zunino, 1986; Boscagli, 1988; Osti, 1991; Clevenger *et al.*, 1992; Fig. 7), after which the couples separate (Mustoni, 2004).

Fig. 7. Annual cycle scheme of brown bear in the Alps (from PAT and Servizio Foreste e Fauna, www.orso.provincia.tn.it)



For the rest of the year, adults are solitary, a behaviour that is regulated by scent markings. In winter, the bear faces adverse environmental conditions, due to low temperatures and consequent reduction of trophic resources, especially in the presence of snow. These factors create a negative energy balance for the brown bear, so maintaining metabolic functions would require more calories than those ingested (Kaczensky, 2000; Zibordi, 2017). For this reason, after the autumnal intense feeding phase, called 'hyperphagia', the species spends the winter inside a den or a retreat (small natural cave) in a state of inactivity, which it can suspend at any time (Folk *et al.*, 1976; Nelson *et al.*, 1983; Mustoni, 2004). The winter resting period of the bear is therefore not true hibernation, but torpor, a specialized seasonal reduction in metabolism, during which males may lose 22% of their body weight, while females may lose as much as 40%, but almost all of the weight lost is fat mass (Mustoni, 2004). Despite these physical variations during the year, the bear remains metabolically healthy. This is due to a change in its intestinal microbiota as a result of the change in diet; in fact, according to Sommer *et al.* (2016), the summer microbiota not only consists of a greater bacteria diversity than the winter one, but its composition promotes adiposity without compromising glucose tolerance. On the other hand, the winter microbiota, with a high level of succinate, suggest a reduced glucose utilization and increased gluconeogenesis during hibernation, when bears mobilize lipids as survival strategy, accompanied by reduced glucose utilization. Physiological activities, such as eating, drinking, defecating and urinating are interrupted during torpor. Despite this, the waste products of metabolism do not accumulate. For example, urine is reabsorbed from the walls of the bladder, and its components are recycled from the liver into aminoacids. Furthermore, even if the bear does not drink for months, through the consumption of fat, water recovery is carried out internally (Zeni, 2016). The period spent in the winter den can be very variable; e.g. in the Alpine environment it generally begins between mid-November and early December and ends in March (Daldoss, 1981).

Embryo development is discontinuous. Initially, it remains free in the womb (Mustoni, 2004). Then, shortly after the start of torpor it implants in the uterine mucosa and continues to develop (Mustoni, 2004; Friebe *et al.*, 2014). This phenomenon, called 'delayed implantation' or 'embryonic diapause', regardless of copulation date, allows cubs (typically 1-3) to be born in January-February, where they are protected in the den (Mustoni, 2004). They will remain with their mother for 15-17 months (Dahle & Swenson, 2003), spending a second winter together in the same den. By the following spring they will have acquired enough knowledge to live independently. Once they leave their mother, the cubs stay together for several months before separating (Mustoni, 2004).

1.2.6 HISTORY IN EUROPE AND THE ITALIAN ALPS

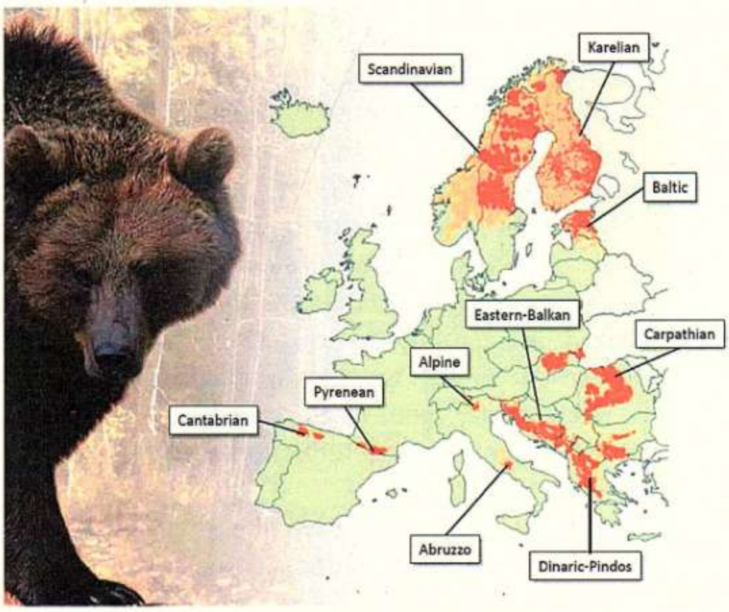
The brown bear has shared its European habitat with modern humans for about 46 000 years. However, in the last two centuries, with the industrial revolution and increase in human population, the bear has been severely limited to very small areas, seriously threatening most populations (AA. VV., 2010a). Thus, this species has become progressively extinct over most of its European range due to deforestation and continuous increase of farmland accompanied by fragmentation of remaining natural habitats (especially Alpine forests), as well as legal and illegal hunting by humans (Dupré *et al.*, 1998). Legislative measures and conservation efforts started to be effective in the 1960s and created the basis for its return into

formerly inhabited regions in northern Europe (Pulliainen 1997; Swenson *et al.* 1995; Kaczensky *et al.* 2012).
In continental Europe, the brown bears occur in 22 countries. Based on the existing data on distribution, as well as a range of geographical, ecological, social and political factors these can be clustered into 10 populations (Chapron *et al.* 2014; Table 2; Fig. 8) The Scandinavian, Dinaric-Pindos, and Cantabrian populations have recorded a clear numerical increase in recent years. All population ranges have been relatively stable or slightly expanding (McLellan *et al*., 2017).

Table 2 Bear population ranges in Europe (McLellan *et al*., 2017; www.balcanicaucaso.org)

Population	N° of individuals	Range
Carpathian	> 8 000	stable
Scandinavian	> 3 400	expanding
Dinaric-Pindos	3 040	expanding
Baltic	~700	stable
Eastern Balkan	~670	stable
Karelian	~400	stable
Cantabrian	~200	expanding
Alpine	~80	slightly expanding
Appennine	~50	stable
Pyrenean	~50	stable

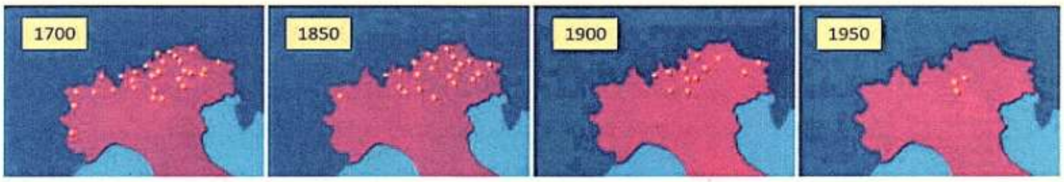
Fig. 8. European distribution of the brown bear. Red: commonly reported; orange occasionally reported (www.ec.europa.eu)



Knowledge of the current connectivity among populations of bears across Europe and national borders is scarce. (Tammeleht *et al.* 2010). In addition, their demographic history in the northern part of the continent is characterized by drastic reductions and subsequent increases in the size of populations ('bottlenecks'), due to human activities (Sørensen *et al.,* 1990; Swenson *et al.,* 1995; Ermala *et al.,* 2003; Danilov 2005). These fluctuations in the number of individuals could have negative consequences; for example, reducing their general level of genetic variability and thus their long-term survival (Relethford, 2013). For this reason, an assessment of the general levels of genetic variability within these brown bear populations is recommended.

Regarding northern Italy, in the 1600s, the bear was still abundant and widely distributed over the entire Alpine area and even in large forests of the Prealps and the Po Valley. Population decline coincided with an increase in deforestation for farming at the end of the 1700s. In the following century, increased access to previously remote wilderness areas of the Prealpine and Alpine mountains, and direct persecution by farmers and hunters, caused the extinction of local populations, commencing in the western Alps (Fig. 9). In 1939 the brown bear became legally protected, but poaching continued, further reducing the number well below the 50-90 individuals threshold, considered the minimum viable population size for this species in the Alpine environment (Schröder, 1992). The

Fig. 9. Historical distribution of the brown bear (*Ursus arctos*) in the Italian Alps (Mustoni *et al.,* 2003a)



bear went extinct in most areas of the Italian Alps in the first half of 1900s, with low numbers persisting in the upper valleys surrounding the Adamello-Brenta and the Cadria mountains, Province of Trento (Castelli, 1935; Pedrotti, 1972; Daldoss, 1976; Oriani, 1991). In 1997 only three animals were reported alive on the Brenta massif (Lande, 1988; Mustoni *et al.,* 2003a). Some evidence suggests that direct persecution by farmers to protect livestock or beehives, and by hunters for sport or money (bounties were paid in most provinces over several decades) was the main factor responsible of the dramatic population reduction. Part of this decline might be also attributed to fragmentation of suitable habitat caused by the construction of roads and structures for tourism in particular in the upper Alpine valleys. Demographic stochasticity, genetic drift and high levels of inbreeding may have further contributed to the drastic reduction in the number of individuals (Mustoni *et al.,* 2003a).

As mentioned above, in order to save the small nucleus present on the Brenta Dolomites from extinction, in 1999 the Adamello-Brenta Natural Park initiated the Life *Ursus* project.

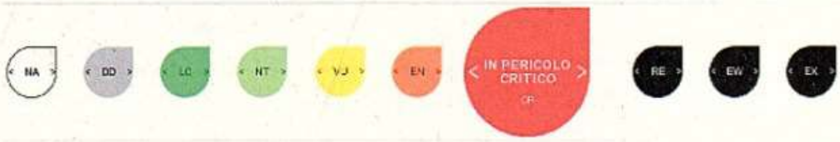
1.2.7 CURRENT LEGAL STATUS

The brown bear is considered a species of 'least concern' on the global IUCN red list as it is very abundant, with expanding populations over much of its global distribution, especially in Russia and North America (Alaska and Canada; McLellan *et al.*, 2017).

However, due to the many small and fragmented populations, at the European level the species is protected by several different laws, such as:

- **Berne Convention** (1979), which places this carnivore among the strictly protected species (Teofili, 2006; AA. VV., 2010b)
- **Habitat Directive** (1992), which includes the brown bear among the species of community interest requiring strict protection (AA. VV., 2010b), and declares that States must ensure the monitoring of its conservation status. Furthermore, its conservation requires the establishment of Sites of Community Importance (SIC) (Teofili, 2006).

In Italy, on the other hand, the brown bear is a 'critically endangered' species on the national IUCN red list as it is represented only by two isolated populations: subspecies *U. arctos marsicanus* in the central Apennines and subspecies *U. a. arctos* in Trentino.



Moreover, the species is protected in Italy by the **Wildlife Act** (1992) that places it in a group of particularly protected species, for which hunting is prohibited and voluntary killing is sanctioned (Teofili, 2006; AA. VV., 2010b).

1.3 CONSERVATION OF THE BROWN BEAR IN TRENTINO

The conservation of the brown bear is a major objective in wildlife conservation strategies for biological and ecological reasons (Teofili, 2006). All large predators (such as bear, wolf and lynx) constitute a fundamental link in the ecological relationships that act within biocenosis and the survival of these species is therefore a key to the conservation of fundamental ecological mechanisms (Davoli, 2015). In addition, a strategy for the conservation of species such as the bear, characterised by very wide spatial requirements and considerable ecological needs, can be an important factor in the conservation of large geographical areas encompassing different habitats. In this sense the bear constitutes an 'umbrella species', and its conservation helps, as a consequence, the stability of the ecosystems. This species is also a useful indicator for measuring their overall ecosystem functionality, testifying that the forests where they live have high quality and quantity of food resources suitable for supporting large carnivores (Davoli, 2015; Teofili, 2006; Zibordi 2017). It is precisely for these reasons that, given the sharp decrease in the number of bear populations in the Alps from about 1700s to the 1900s, the Life *Ursus* reintroduction project was proposed by the Adamello-Brenta Natural Park (AA. VV., 2002; Mustoni *et al.*, 2003a; Zeni, 2016).

1.3.1 THE LIFE *URSUS* PROJECT

The Life *Ursus* project was first proposed by the Adamello-Brenta Natural Park (PNAB) in 1995, and formally approved and financed by the European Union in 1996. It was implemented in collaboration with the Autonomous Province of Trento (PAT) which provided constant support from an organizational, political and financial point of view, and the National Wildlife Institute (INFS: the scientific body of the Italian Ministry of the Environment, now known as ISPRA – the Italian Institute for Environmental Protection and Research) that deals with the technical-scientific aspects.

This type of project was not new in Europe; in fact, there had been two other attempts at bear reintroduction, both in Austria (1989-1993) and in the Pyrenees (1996-1997), but these projects failed, reportedly due to insufficient publicity on the media and lack of local involvement, which led to an intolerance towards the species and resulted in poaching (Mustoni *et al.*, 2003a; Davoli, 2015). In Trentino, in the second half of the 1900s three attempts were made to restock this population, but these 'experiments' also failed because reintroduced animals were imprinted on humans (AA. VV., 2010a). Therefore from 1996 to 1998 a detailed Feasibility Study was carried out to examine the social, economic and biological aspects of the project and the likelihood of success (Mustoni *et al.*, 2003a; Davoli, 2015). Among wild species, the bear has one of the greatest emotional impacts on humans and the conservation of this species often conflicts with human activities, such as fruit cultivation and livestock farming. Therefore, the feasibility study was particularly important for investigating the acceptance and sharing of conservation objectives for such a controversial species, a fundamental condition for achieving them (Davoli, 2015; Zibordi 2017).

The project aimed to release in Trentino ten bears captured in Slovenia (AA. VV., 2002; DeBarba *et al.* 2013; Davoli, 2015). The short-term objective was to reconstitute a Minimum Vital Population of bears in this area, which according to studies on the suitability of the habitat and on the ecological needs of the species

was around 50-90 individuals, distributed in an area of 6500 km^2, with the Adamello-Brenta Natural Park as the *core area* (645 km^2) (AA. VV., 2002; Mustoni *et al.*, 2003a; Davoli, 2015). The long-term objective of this project was to connect the Trentino and Slovenian populations (Zeni, 2016). So, the realization of the reintroduction involved a prolonged effort over time.
The structure by age, sex and origin of the founders (Tab. 3) aimed to:

I. increase the probability of rapid growth of the bear nucleus;
II. decrease the risk of excessive dispersion;
III. limit the risk of behaviour unacceptable for human populations;
IV. ensure maximum genetic variability, minimizing the risk of releasing related individuals.

Table 3 Structure by sex and age of the founder population, Life *Ursus* project (AA. VV., 2010a)

Name	Sex	Age (yr.)	Year of release
MASUN	Male	3-4	1999
KIRKA	Female	3	1999
DANIZA	Female	5	2000
JOZE	Male	6	2000
IRMA	Female	6	2000
JURKA	Female	4	2001
VIDA	Female	3	2001
GASPER	Male	4	2002
BRENTA	Female	3	2002
MAYA	Female	5	2002

A high proportion of females was considered preferable, both because females have more limited movements, and because an unbalanced sex ratio in favor of females was believed to support the chances of an increase in the initial nucleus. Individuals two to six years old were chosen, young enough to adapt easily to their new surroundings, but old enough to have completed their body development which allowed them to be equipped with a radio-collar (Davoli, 2015).

1.3.2 POPULATION MONITORING

All 10 bears released in the context of the Life *Ursus* project were equipped with a radiocollar with a 36-month battery life and two radio-emitting eartags allowing them to be monitored in real time up to 22 months during the crucial phases of adaptation to their new habitat and to prevent conflicts with humans. In many cases, the detachment of the radiocollar occured before the 36 months guaranteed by the battery, thanks to a mechanism called *drop-off* designed to allow the collar to widen and avoid the suffocation of the growing animal (AA. VV., 2010a; Fattori *et al.*, 2010).

Such 'biologging' is widely used in wildlife biology to acquire data on the position or physiological parameters of marked animals, even for very elusive species. Through this technique it is also possible to study the space used and the distances traveled daily by single individuals (Boyce *et al.*, 2010), the activity rhythms and population dynamics (Cagnacci *et al.*, 2010; Fattori *et al.*, 2010; De Barba *et al.*, 2013). A study of biologging data of the bears released in Trentino during the Life *Ursus* project showed that each individual had a distinct spatial behavior that was not associated with sex or age. However, land use changed with season for all individuals (Mustoni *et al.*, 2003b).

Biologging confirmed that one founder female died in an avalanche during the first winter after release. One male was presumed dead in 2001 after its signal was lost, and one female emigrated to Austria in 2002. The remaining seven founder individuals survived and adapted well to the new environment. In early phases after release, an exploratory activity was noted for all of them. Biologging monitoring also showed that all founder bears, with the exception of one female, distributed themselves in the first years after the release within the boundaries of the area identified by the Feasibility Study (AA. VV., 2010a; Zeni, 2016; Adamello-Brenta Natural Park website).

Biologging was the main monitoring method from the beginning of the project to 2003, when the last bear dropped its radiocollar, after a total of 51 months. After 2003, biologging has only been used to monitor 'problematic' bears; that is, those habituated animals with no fear of humans, that roam or prey on livestock near residential areas, or risk becoming dependant on trophic resources of anthropic origin. The problematic level of such individuals increases with the frequency of dangerous behaviours. A bear considered 'harmful', i.e. causing damage to crops, livestock or beehives, can over time become 'dangerous' if it poses a high risk of injury for humans. Therefore, these bears need individual monitoring and, in specific cases, when re-education is not possible, they may be placed in captivity or euthanised. This decision can be taken only with the authorization of the Ministry for Environment, Land and Sea Protection of Italy (MATTM) and consultation with the Italian Institute for Environmental Protection and Research (ISPRA), on condition that there are no alternative solutions and that such culling does not affect the maintenance of the population in a good state of conservation (DPR 357/97 art. 11; AA. VV., 2010b; Frapporti *et al.*, 2014; Groff *et al.*, 2019). The two authorized bear culls in Trentino (the first, of a female bear named 'Daniza' in 2014, that accidentally died during capture operations following aggressive behaviour, and the second, the voluntary culling of the bear named 'KJ2' in 2017, following a second attack on humans) have been much criticized and debated both locally and internationally. For this reason, biologging is very important as a method to ensure proper wildlife management and to avoid bears becoming dangerous.

Starting from 2003, genetic monitoring became the principal method for obtaining information on the founder population and their descendants. This method is based on individual genotyping of the DNA extracted from non-invasive biological samples collected in the environment, mostly hair and feces, without the need to manipulate the animal. Occasionally tissue, blood, and bones are collected during capture operations or from bear carcasses (see Methods for details). From 2003 to 2016, ISPRA was responsable for this analysis for the Trentino population; however, since 2017 genetic monitoring has been carried out in the animal genetics laboratories of the Conservation Genetics Research Unit of the Fondazione Edmund Mach (CONGEN-FEM), in collaboration with ISPRA. These tasks were transferred to FEM when genetic monitoring began to be used to compensate farmers for damage to their herds or crops and, thus results were needed within two weeks rather than annually.

Genetic monitoring has made it possible to obtain data on the demography, reproduction and distribution of individuals in the Trentino bear population (Fattori *et al.*, 2010; De Barba *et al.*, 2010; De Barba *et al.*, 2013). Following the first reproductive event after the start of the Life *Ursus* project (recorded via *camera-trapping*), genetic analyses of environmental samples showed that all cubs born between 2002 and 2005 were fathered by a single male, and that two litters derived from a mating between father and daughter, one in 2006 and one in 2008. The population grew rapidly, due to high survival and reproductive rates (De Barba *et al.*, 2013; Zeni, 2016), reaching an annual growth trend of 12% of the total population consistency in the period 2015-2019. In 2019 there were an estimated 77 individuals (Groff *et al.*, 2020; Adamello-Brenta Natural Park website). Individual genotyping is also combined with traditional surveys of tracks or rub-trees to provide estimations of the distribution and behaviour of the brown bear (AA. VV., 2010a; Groff *et al.*, 2019).

1.3.3 GENETIC VARIABILITY

Genetic variability is the measure of polymorphism at particular DNA markers of individuals of the same population or species. This diversity is mainly due to mutations, which lead to the formation of new alleles and to the genetic recombination processes during meiosis that create new allelic combinations in subsequent generations. Mutations can occur at any time in the life of organisms and are completely random events. More precisely, a population of individuals with different genotypes, defined as the genetic composition of an individual consisting of all the alleles present for each locus studied, and a considerable number of alleles per locus, is considered to have good levels of genetic variability (Relethford, 2013).

High levels of natural genetic diversity are essential for wildlife populations to evolve in response to changes in abiotic parameters (non-living environmental factors, such as fire, light, moisture, temperature, wind; an example is the case of the current global climate change due to human activities that is forcing species to adjust their ranges to follow their referred habitat (Hughes, 2000)), and biotic parameters (changes in ecological relationships and interactions with other organisms; J. I. Muoghalu, *unpublished*). Therefore, there can be no adaptation, or changes in the allele frequencies of populations, if there is no individual genetic variation. In fact, evolution must have this 'raw material' on which to operate (Frankham *et al.*, 2006; Primack *et al.*, 2013). There's only one alternative to adaptation: death, if we're talking about a single organism, or extinction, if we're