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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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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).

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).

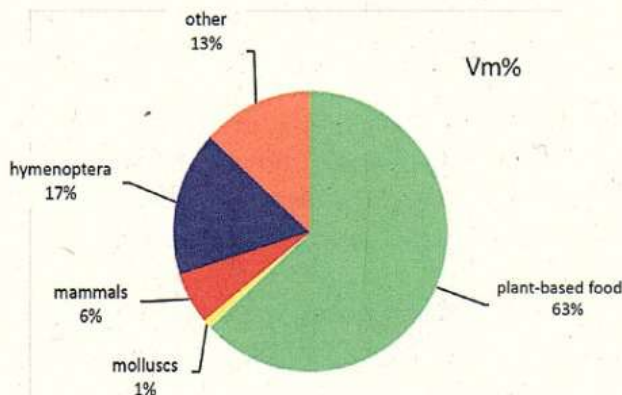
Fig. 5. Brown bear hair. From the left: silky hairs of the underpaws, wad hairs and giarra hairs (Mustoni, 2004).

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

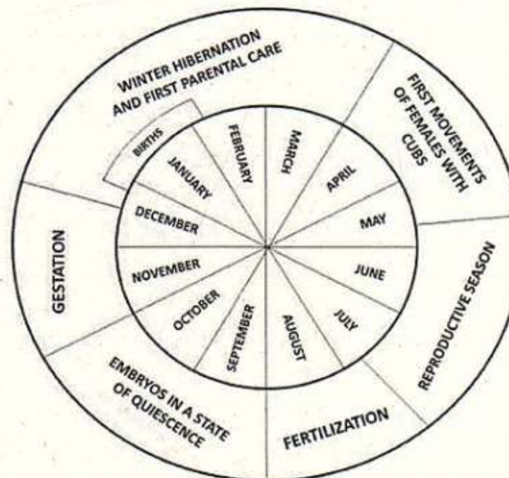
A very important ecological requirement for the brown bear is the availability of vast territories, with a high environmental diversity to provide both the necessary food resources, refuge areas and mates (Couturier, 1954; Boscagli, 1988). Although the preferred habitat of this species is deciduous forests 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)

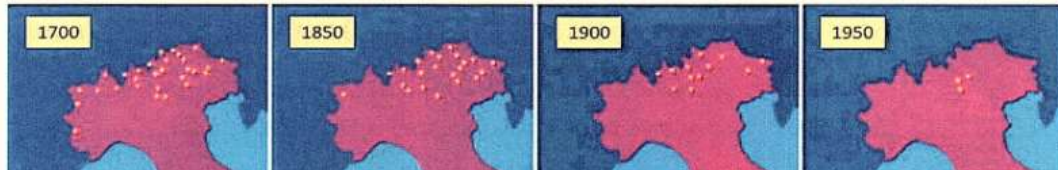
Population	N° of individuals	Range
Carpathian	> 8 000	stable
Scandinavian	> 3 400	expanding
Dinaric-Pindos	3 040	expanding
Baltic	~700	stable
Eastern Balkan	~670	stable
Karelian	~400	stable
Cantabrian	~200	expanding
Alpine	~80	slightly expanding
Appennine	~50	stable
Pyrenean	~50	stable

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.

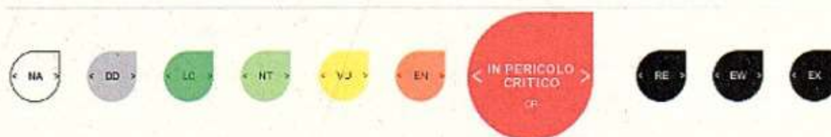
1.2.7 CURRENT LEGAL STATUS

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

talking about whole population or species. Thus, population genetics investigates, with the tools of molecular biology, statistics and ecology, the diversity within and between populations, to promote sustainable management of biodiversity. In this way the principles of genetics are applied to populations. This approach is necessary in order to evaluate the evolutionary potential and the permanence of a species, or a population, in the long term.
Numerous studies have shown that high levels of population genetic variability increase the productivity and stability of ecosystems (Worm *et al.*, 2006; Tilman, 2012; Soulè 2013; Willoughby *et al.*, 2015), as well as the probability of survival of species (Reynolds, 1999; Walling *et al.*, 2011; Relethford, 2013). These results are mainly due to an increased individual fitness when high levels of genetic diversity are present (Frankham *et al.*, 2002). In addition, it is well known that high levels of genetic variation increases individuals' resistance to pathogenic organisms (Daszak *et al.*, 2008).
The levels of variability within populations are measured by the use of genetic markers, that is, regions of DNA whose exact position on the chromosome is known and which can be described by observable variations. Single individual or species can be identified by defining the alleles within the set of genetic markers considered that define those individuals or species. The choice of the type of marker to use depends on the purpose of the research. In cases where it is necessary to determine species, mitochondrial DNA (mtDNA) is widely used as a genetic marker, which is a DNA of maternal origin and present in thousands of copies into cells; mtDNA evolves slowly and therefore, is useful for identifying species. Recently the marker of choice for population genetics studies are SNPs (*Single Nucleotide Polymorphism*), thousands of which are found throughout the genome, in both coding and noncoding regions. Non-neutral SNPs are subject to selection, which can provide information about the effect of natural selection on a given population and its evolutionary potential (Frankaham *et al.*, 2006; Relethford, 2013; Nielsen E. *et al.*, 2020). However, this type of marker requires high quantities and qualities of starting DNA, i.e. DNA obtained from samples collected with an invasive method (for example tissue or blood), and for the brown bear, are not currently sufficiently developed for genotyping non-invasive samples. Therefore, in the present study I used more traditional microsatellites, or STRs, neutral, highly polymorphic genetic markers that allows individual genotyping from feces and hair. In addition, STRs are relatively widely dispersed throughout the genome, so threy are assumed to be representative of the entire variability for a given individual. For these reasons microsatellites are widley used in ecological, evolutionary and conservation research (Davoli *et al.*, 2013; Relethford, 2013; De Barba *et al.*, 2017).

Long-term genetic monitoring is particularly important following wildlife restocking, as well as reintroductions, to assess the success of such programmes and to ensure the probability of population growth and persistence (Casas-Marce et al., 2013; Miller-Butterworth *et al.*, 2021). Often a small number of founding individuals are released during the reintroductions of large carnivores, as in the case of the Life *Ursus* project. These reintroduced species can suffer from a genetic 'founder effect' since the new population grows from a small nucleus of released individuals that are spatially separated from their original population, resulting in a reduction of

their genetic variability, mainly due to genetic drift and **'inbreeding depression'** (Frankaham *et al.*, 2002; Jamieson, 2010).
'Genetic drift' can also contribute to the reduction of genetic variability in reintroduced populations, that is, the random fluctuation of allele frequencies within genes over time, which can result in the fixation of certain alleles and loss of others. The smaller and more isolated these populations are, the more likely it is that drift will substantially modify their allelic frequencies, generating extreme values (England *et al.*, 2003; Relethford, 2013; Honnay, 2013).
Populations that have had an important reduction in the number of individuals followed by a recovery in size may also suffer the so-**called 'bottleneck effect', or** considerable fluctuations in allele frequencies, resulting in high levels of inbreeding depression and genetic drift (Dunn *et al.*, 2011; Casas-Marce *et al.*, 2013; Frankaham *et al.*, 2002).
Genetic diversity can be mantained monitoring these populations constantly over time. Based on the results of genetic analysis it will therefore be possible to propose programs to maintain high levels of variability within these populations, i.e. promoting the connection between them to increase gene flow (an example is ecological corridors), or carrying out restockings through the translocation of some individuals (Casas-Marce *et al.*, 2013; Miller-Butterworth *et al.*, 2021). Therefore, in contrast to what was thought until a few years ago, when the only parameter that was evaluated was the total number of individuals present within a population, preserving genetic diversity is now seen as a way of increasing viability of wild populations, especially after a reintroduction or restocking project (Reynolds et al., 1999; Hemphill et al., 2020; Willoughby *et al.*, 2015).

The first study of the genetic variability of the brown bear in Trentino was carried out by De Barba *et al.*, in 2010, ten years after the reintroduction program. As part of this study, biological samples obtained by opportunistic sampling and *hair-traps* (see Methods for details) between 2002 and 2008. Ten microsatellite loci were analysed, and the main indices of genetic variability were estimated per year (Table 4).

Table 4. Estimates of genetic parameters for the Trentino brown bear population from 2002 to 2008 using 10 microsatellite loci; H_e, expected unbiased heterozygosity; H_o, observed heterozygosity; A, allelic richness; F, mean inbreeding coefficient from pedigree; K, mean kinship from pedigree; F_{is}, average individual inbreeding coefficient within each population; Ne demo, effective population size computed using the demographic method. Levels of significance (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$) are shown for the paired t-test comparison of H_e and A of the 2008 population to the founders, and for F_{is} (De Barba *et al.*, 2010).

Year	H_E	H_O	A	F	K	F_{IS}	$N_{e\ demo}$
Founders	0.79	0.76	5.41			0.039	
2002	0.78	0.79	4.99	0	0.075	−0.014	2.75
2003	0.76	0.79	4.72	0	0.095	−0.038	2.78
2004	0.76	0.81	4.48	0	0.106	−0.079	2.84
2005	0.74	0.83	4.48	0	0.121	−0.116	3.08
2006	0.75	0.83	4.49	0.010	0.124	−0.115**	2.84
2007	0.75	0.86	4.38	0	0.121	−0.155***	3.03
2008	0.74*	0.85	4.55***	0.017	0.134	−0.152***	NC

De Barba *et al.*, (2010) concluded that the genetic variability of the brown bear population in Trentino generally decreased over the study period, especially with regards Expected heterozygosity (H_e) and Allelic richness (A), while Mean inbreeding coefficient (F) and Mean kinship (K) increased.

2. AIM OF THE THESIS

This study aimed to evaluate changes in genetic variability of the brown bear population in Trentino over the two decades following its reintroduction. This is an important assessment in light of the demographic history of this population, since: I) it may have suffered a founder effect, as only ten individuals were translocated in Trentino from their original population in Slovenia; II) during the years after release no immigration event has been recorded, suggesting no gene flow has occurred between these two populations; III) reproduction between related individuals has occurred since the start of Life *Ursus* project, possibly causing inbreeding depression (de Barba *et al.*, 2010; Zeni, 2016). Previous studies of other brown bear populations and of other reintroduced species have reported a decrease in the level of genetic variability over several generations, due to an increase in inbreeding depression and genetic drift, caused by isolation and absence of gene flow, leading to a decrease in fitness and/or their extintion (Reynolds, 1999; Taberlet *et al.*, 1999; Woodworth, 2003; Benazzo *et al.*, 2017; Hemphill *et al.*, 2020; Lorenzana *et al.*, 2020). Therefore, I also expect a decrease in genetic diversity in this case study. In addition, this database is also useful for testing the most appropriate method to detect genetic variability in small and dangered populations. For this analysis I considered all 174 bear genotypes obtained from the beginning of the Life *Ursus* project (including those of the founder individuals); that is, from 1999 to 2019. I divided the genotypes based on four criteria, in order to test when changes in genetic variability were detectable. First of all, I extended De Barba's study, estimating population indices per year so I could compare my results with hers. I also repeated this analysis twice: once with the same 10 microsatellites used by De Barba *et al.* (2010), and once with the 15 microsatellites currently employed, to test whether more microsatellites resulted in different estimates. Then I divided the entire database into five year age cohorts; that is groups of individuals that were born in the same five year time-frame and therefore, share the same life-history phases (five years is the approximate generation time of the brown bear). Next, I divided the genotypes into generations based on parental types, considering the 'founders' as 'F0', to study the genetic variability lost between generations. Finally, I mimicked a typical conservation genetics sampling regime for large carnivores, randomly 'sampling' thirty individuals every three years to test if these results were comparable to the other analyses listed above. For each subdivision I estimated the main indices of genetic variability and compared these results. I also used the results to predict future scenarios for this population if the conditions continue to mirror the current ones (i.e. no immigration events, mating between relatives), or if additional animals are released. Since interventions for the management and

conservation of the species may be implemented, I propose solutions to maintain or increase the genetic variability of the population. Finally, I discuss possible future monitoring strategies, highlighting advantages and disadvantages.

3. MATERIALS AND METHODS

All the phases of this study (from the collection of samples to laboratory analysis and database creation) were carried out following the technical protocol 'PACOBACE': Interregional Action Plan for the Conservation of the Brown Bear in the Central-Eastern Alps, which represents the reference document for the management of the brown bear for the regions and autonomous provinces of Italy.

3.1 COLLECTION, CONSERVATION AND STORAGE OF BIOLOGICAL SAMPLES

There are various non-invasive methods of sampling bear hair and feces and this choice depends on both the environmental context and the objectives of the monitoring. The sampling design can be 'opportunistic', if the samples are collected during the routine activities of wildlife personnel surveying the territory; or 'systematic' if sample collection is carried out at regular intervals, for a limited period of time and on a predefined grid, such as (in the case of the brown bear) through the use of *hair-traps* with scented baits or rub-trees.
For this study two kinds of biological sample were available, each of which require different methods of collection and storage. All samples were collected by wildlife technicians of the Large Carnivore Section, Wildlife Service, Autonomous Province of Trento.

- Hair - collected with hair traps during systematic sampling. Hairs were collected with sterile tweezers and inserted in new paper bags that were then placed in a plastic ziploc bag containing silica gel, sealed and stored in a cool and dry place.

- Feces - collected from the ground during opportunistic sampling. Portions of these large fecal deposits were sampled to maximize the probability of finding genetic material, that is from the extremities and surfaces. Three different collection protocols are followed:

I. A portion of excrement, collected with a single use wooden tongue depressor, is placed in a wide mouth 25 ml square polyethylene bottle with 75% ethanol that can be stored at room temperature or -20°C.
II. About 250 mg of excrement is collected with a single use wooden tongue depressor and placed in a 5 ml sterile tube containing the first buffer used in the DNA extraction (SLX-Mlus Buffer, E.Z.N.A. ® Stool DNA Kit). This method has proved particularly effective for the preservation of the sample, both at room temperature and at -20°C, and speeds up the extraction process.
III. A GenoTube (Thermo Fisher Scientific ®) swab is wiped over the surface of the stool, in order to collect as many host cells as possible avoiding the collection

dietary material. This is a rapid method for collecting sample and improves the quality of the extraction. The sample is stored at room temperature.

Each sample was identified by a unique code containing the collector, the year of collection, the number of the sample and the presumed species in Italian; for example:

UUD1801O

means this sample was collected by Udine University (UUD) in 2018 (18); it was the first sample for that year (01) and presumably belongs to a bear (O = 'orso').

In addition, each sample was accompanied by a datasheet reporting the date and time of collection, GPS coordinates, the field technicians responsible for the collection and additional observations.

3.2 LABORATORY PROCEDURES

3.2.1 SAMPLE PREPARATION

From each hair sample about 15 hairs with bulbs were selected and placed in a sterile 1.5 ml Eppendorf.

All fecal samples were placed in the freezer at -80°C for at least 72 hours, to reduce the biological risk of pathogens potentially transmitted by the feces of certain carnivores (usually red fox, wolf, domestic dog) such as *Echinococcus multilocularis*. The preparation of fecal samples varied with method of collection:

I. For faeces preserved in ethanol: under a biological hood, about 250 mg was taken from the pellet where the genetic material is most concentrated: on the outer surfaces or in the initial and final parts of the pellet. The subsample was placed into a 1.5 ml Eppendorf for DNA extraction.
II. The samples stored in lysis buffer (SLX-Mlus Buffer) were placed in 5 mL tubes when collected.
III. For samples collected with a GenoTube (Thermo Fisher Scientific®), the swab was cut into two subsamples with sterile scissors, each of which was inserted into a sterile 1.5 ml Eppendorf. DNA was initially extracted from only one of the two subsamples, while the other was stored at -20 ° C for possible further extraction.

3.2.2 DNA EXTRACTION PROTOCOLS

The extraction phase is critical, separating the genetic material from other cellular components, and purifying the sample of potential inhibitors that could interfere in subsequent analyses. This phase involves three different steps:

I) **enzymatic lysis** breaks down cellular and nuclear membranes and then, denatures histonic proteins, releasing the DNA molecules. To each subsample in a 1.5 ml Eppendorf 20 µl *proteinase-K* was added and

activated by an incubating in the Eppendorf in a thermo-mixer at 56-70°C

II) **DNA precipitation** thanks to substances such as ethanol and silica; in the presence of ethanol the DNA binds to the silica, while all other cellular components are removed;

III) **solubilization** of DNA, thanks to low salinity solutions that remove the DNA from the silica membrane, causing it to precipitate in an aqueous solution.

Genetic material was extracted manually if the number of samples was less than 40 using two different kits depending on the kind of sample: for feces and urine the 'EZNA® Stool DNA kit' (Omega Bio-Tek, Norcross, GA, USA) was used; for hair and feces collected with the GenoTube swab the 'QIAamp® DNA Investigator Kit' (Qiagen, Inc., Valencia, CA, USA) was implemented. Manual sample extraction with these kits uses DNA purification via *silica gel/membrane*. When there were more than 40 samples extraction was carried out in 96-well plates with the KingFisher Flex Purification System (Thermo Fisher Scientific®) using the 'Mag-Bind ® Blood & Tissue DNA HDQ 96 Kit' (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's instructions. Automated extraction with the KingFisher uses charge-switch DNA purification. Lysis is carried out as described above, but the isolation phase uses paramagnetic particles, coated with molecules that change their charge according to pH. Paramagnetic particles become attractive with acid pH, so they become positively charged and interact only with DNA, that is a molecule with negative charge. Then, the bond between the paramagnetic particles and the DNA molecules is created. Some subsequent washes with acid pH buffers remove the other molecules and contaminants. The third phase is characterized by higher pH washes (8.5) that cause DNA to detach from the paramagnetic particles. At the end of the extraction I obtained 100-150 µl of solution containing the DNA to be used in the amplification phase. To detect possible contamination at least one negative control (K-EXT) was added into each extraction protocol, that is (for manual extraction) an Eppendorf or (using the Omega kit) the last well containing only the reagents and treated in the same way as the other samples.

3.2.3 AMPLIFICATION OF MICROSATELLITE (STR) LOCI

The low quantity, often degraded, DNA extracted from non-invasive samples, is often not sufficient to carry out direct sequencing and must first be amplified through the polymerase chain reaction (PCR, Mullis *et al.*, 1986). PCR is an enzymatic reaction that allows to make in vitro, faster than in nature, the process that leads to duplication, thus obtaining millions of copies of the DNA sequences that we want to study, producing enough of them to be analyzed. Following the PACOBACE protocol, brown bear genotyping requires the amplification of 10 microsatellite loci and a sex determination locus (AMG). By amplifying groups of loci **simultaneously ('multiplex', MPX), the amount of** DNA needed is minimized (Taberlet *et al.*, 1999). If the analysis of the first 10 loci (MPX A 6 loci) and B (5 loci) results in a new genotype, that is a genotype not previously recorded, an additional

MPX (C) of 6 loci, including the sex determination locus SRY, must be amplified and analyzed to confirm the identification of a new individual.
In the genotyping of non-invasive samples there is an increase in the error rate mainly due to *allelic drop-out* (failure to read one of the two alleles that makes a heterozygous individual apparently homozygous), and *false alleles* (amplification errors due to poor quality of the starting material; in this case a homozygous individual can be mistaken for a heterozygote). Therefore, to obtain accurate genotypes from poor quality/quantity DNA, the 'multi-tube approach' has been proposed, which involves amplifying each sample several times, in independent replicates. Genotypes are obtained by comparing the results of each replicate. Here, each sample was amplified a minimum of four times and the result at each individual locus was considered reliable if at least four identical repetitions were obtained (Taberlet *et al.*, 1999). For each amplification contamination was evaluated using a negative control (sterile water) in each PCR (K-PCR).

3.2.4 INDIVIDUAL GENOTYPING AND GENETIC TAGGING

For the typing of microsatellite loci 1 µl of PCR, previously diluted (1:20) with water, has been loaded with 10 µl of formammide (Hi-Ti Applied Biosystem) and 2.1% GS500LIZ dimensional standard (Applied Biosystems, Foster City, CA, USA), on the ABI 3130XL DNA sequencer, with 96 capillaries of 50 cm and POP07 polymer, available at FEM sequencing platform. Automatically processed data were manually reviewed and verified using the GeneMarker® software. Once the multilocus genotype were obtained, the identification of the individual (genetic-tagging) was carried out using GenAlEx® software. This software compares the multilocus genotype of each sample with those of individuals already registered in previous monitoring years. For each locus, the values of the two alleles were analyzed and the probability of compatibility between the database genotypes and the identified genotype was defined. Genetic tagging was carried out taking into account all 10 microsatellite loci of MPXA and MPXB. In case of mismatch with the genotyped individuals in previous years, the presence of a new individual is assumed and MPXC loci were also analysed.

3.3 DATABASE AND DATA EXTRACTION

I decided to divide the entire database into various groups of individuals, in order to verify which criteria of subdivision is more suitable for detecting trends of genetic variability in this kind of population genetic study.
First of all, I divided the database into years from 2002 (when the first litter from the beginning of the Life *Ursus* project was documented) to 2019, considering all individuals that were alive in each year (Tab. 5). In this way I obtained nineteen subpopulations, one for each year from 2002 to 2019 and one for the 'founders' that were considered a separated subpopulation. In this way, my results could be compared with those of De Barba *et al.*, 2010. In order to compare my results with theirs even more directly, I repeated the analysis twice: once considering the 15

microsatellites currently employed for individual identification, and once considering the 10 microsatellites used by this paper, also to verify whether the use of a greater number of microsatellite loci results in a different estimate of the levels of genetic variability within the population.

Table 5 (N) of individuals considered alive in each year from 2002 to 2019.

YEARS	N
Founder	9
2002	10
2003	11
2004	15
2005	18
2006	22
2007	23
2008	27
2009	30
2010	37
2011	35
2012	45
2013	42
2014	43
2015	53
2016	55
2017	56
2018	72
2019	72

Then, I divided the database into cohorts; that is, groups of individuals that were born in the same time-frame. In this case I defined time-frame as the generation time for brown bear, or 5 years (Pedrotti L. *pers. comm.)*. In this way I obtained five different cohorts, including the 'founder' group (Tab. 6).

Table 6 Groups obtained through the subdivision by cohorts. 'N' is number of individuals that were born in every cohort

COHORTS	Period of birth	N
Founder	---	9
Cohort1	2002-2006	25
Cohort2	2007-2011	26
Cohort3	2012-2016	51
Cohort4	2017-2021	37

The third criteria used was a subdivision by generations, basing on genetic data. I defined as 'F0' the generation composed by the founders; 'F1', individuals whose parents belong to F0 and so on, until 'F3'. I also considered two back-cross generations, that consist of individuals that have parents from two different generations (Tab 7).

Table 7 Groups obtained through the subdivision by generations. 'N' is number of individuals belonging to each generation

GENERATIONS	Parents	N
Founder (F0)	---	9
F1	F0 X F0	24
F1b	F0 X F1	37
F2	F1 X F1	11
F2b	F1 X F2	37
F3	F2 X F2	12

By comparing the results of 'generations' and 'cohorts' it will be also possible to evaluate if the generation time of five years for brown bear is a reliable estimate. Finally, I randomly sampled thirty individuals that were considered to be alive every three years from 2002 on through, mimicking a typical sampling regime for population genetics studies (Tab 8). The founder group and Group1 have fewer than 30 individuals since only 9 and 17 individuals were alive, respectively, in these two groups.

Table 8 Groups obtained through the subdivision by genetic sampling groups. 'N' is number of individuals belonging to each group

GENETIC SAMPLING GROUPS	Period	N
Founder	---	9
Group1	2002-2004	17
Group2	2005-2007	30
Group3	2008-2010	30
Group4	2011-2013	30
Group5	2014-2016	30
Group6	2017-2019	30

3.4 DATA ANALYSIS

Firstly, I carried out some preliminary tests for each of the subdivisions described above to assess if the population was in genetic disequilibrium. Using GENEPOP version 4.7.5 (Rousset, 2008) I tested for Hardy–Weinberg equilibrium for each locus in each population (HWE; exact test based on 1000 as dememorization number, 1000 iterations, and 200 batches for cohorts, 300 batches for generations and the subdivision by years considering 15 microsatellites, and 400 batches for genetic sampling groups and the subdivision by years considering 10 microsatellites used by de Barba *et al.*, 2010), heterozigosity excess (exact test based 1000 as

dememorization number, on 1000 iterations and 400 batches for genetic sampling groups, and 600 batches for all the other subdivisions), and heterozygosity deficiency (exact test based on 1000 as dememorization number, 1000 iterations, and 300 batches for cohorts and genetic sampling groups, 400 batches for generations and 600 batches for the subdivision by years considering both 15 and 10 microsatellites) to evaluate both the alternative hypothesis for those loci that reported a disequilibrium from HWE. For these tests, I estimated P-values by the Markov chain method. Using GENEPOP I also calculated an overall estimate of F_{is} (inbreeding coefficient) weighted for sample sizes (as in Weir and Cockerham, 1984) for every subpopulation to evaluate if the recorded mating between relative individuals have led to negative consequences in terms of homozigousity, and Linkage Disequilibrium for each pair of loci in each population (LD; exact test based on 1000 as dememorization number, 1000 iterations, and 900 batches for generations, 1000 batches for cohorts, 1100 for genetic sampling groups and the subdivision by years considering 15 microsatellies, and 1300 batches for the subdivision by years considering 10 microsatellites used by de Barba *et al.*, 2010), estimating P-values by the Markov chain method. I evaluated the observed (H_o) and expected (H_e) heterozygosity for each locus in each population and overall loci in each population, the average number of alleles per locus (*Na*) and the average effective number of alleles per locus (Ne^{all}) (which is the true diversity of the expected heterozygosity expressed as 1/(1-*HE*)) using GENALEX version 6.5. Allelic richness with rarefaction (that is the value of allelic richness weighted for sample sizes, as in Petit and El Mousadik, 1997) and the number of private alleles within groups were obtained using HP-RARE software (Kalinowski, 2004; Kalinowski, 2005). In order to evaluate the genetic distance between groups, I calculated the F_{st} (Weir and Cockerham 1984), with FSTAT software v. 2.9.4 (Goudet 2003), using Bonferroni corrections for the comparisons within groups. To evaluate if there was an effect of genetic drift I also used FSTAT to calculate the allele frequencies of each locus within groups. Effective population size (N_e) was estimated basing on genetic data; I used the program NE-ESTIMATOR v. 2.1 (Waples *et al.* 2014) to evaluate two single sample estimators, the first one based on Linkage Disequilibrium (Hill 1981; Waples 2006; Waples & Do 2010) and the second one based on heterozygousity excess (Pudovkin *et al.* 1996; Zhdanova & Pudovkin 2008). Then, the levels of significance for the changes in expected (H_e) and observed (H_o) heterozygousity, Allelic richness (A), and F_{is} between each group and the founders was assessed using a pairwise t-test between loci in R software v. 4.1.0. Finally, I searched for signals of a recent population reduction with the software BOTTLENECK v. 1.2.02 (Piry *et al.*, 1999), setting I.A.M., S.M.M. and T.P.M. as mutation models, 12 as variance for T.P.M., and 70 as proportion of S.M.M. in T.P.M. (%). I used 1000 iterations and 'sign test', 'Wilcoxon test' and 'mode-shift' as statistical analysis.

6 RESULTS AND DISCUSSION

With regards preliminary tests, the Trentino brown bear population seems to be in **Hardy-Weinberg equilibrium,** since most loci were in HWE throughout the study period. This trend was similar for all database subdivisions (year – 10

microsatellites, year – 15 microsatellites, cohort, generation and subsampling). However, more loci showed a non-significant excess of heterozygosity in cohort2 (2007-2011), generations F1 and F1b, and group3 (2008-2010); that is, in the years immediately following the release of the founding individuals. HWE was restored in subsequent years. In particular, one locus, 'G10H', reported a slight deficiency of heterozygosity between 2015 and 2018. In addition, analysis by 'year' showed an increase in **linkage disequilibrium (LD)** among loci from 2002-2019 compared to the founder group, considering both 15 and 10 microsatellite loci. This means that the segregation of the analyzed loci tends to be less random over time, causing an association between some alleles of different loci, with a higher frequency than expected, a probable consequence of populations originating from a small nucleus of founding individuals, in which mating is not random (as noted by Strachan & Read, 2004; Allendorf & Luikart, 2007). The **H_o values** remained similar (non-significantly different: 0.67-0.87) from the Founder group througout the study for all database subdivisions, although values were somewhat higher for year – 10 microsatellites (Table 9b). Despite an initial increase, this index is showing a tendency to decrease. **H_e values** (based on HWE) decreased significantly ($P < 0.05$) immediately **after the 'founder' group** for year – 15 microsatellites, cohort 2, generation F1b and subsampling group2, but not for year – 10 microsatellites (Tab 9). **F_{is}** (inbreeding coefficient) shows a significant decrease between about 2004-2010 then a return to founder group levels for all database subdivisions except cohort, although the decrease is more evident in year – 10 microsatellites, a similar but opposite trend to H_o values. These latter two differences are expected since F_{is} represents the fraction of homozigous individuals within a population. **Allelic richness** decreased significantly from the Founder group for all subdivisions immediately following the release of these individuals; a similar trend is also evident for the number of private alleles. Interestingly, the significant and almost immediate decreases noted in H_e values, allelic richness and **number of private alleles** indicates that the founder and bottleneck effects have had important impacts on genetic variability in the Trentino brown bear population, as expected, from population genetic theory (Cornuet & Luikart, 1996; Haeringen *et al.*, 1999; Miller-Butterworth *et al.*, 2021), but rarely shown from natural populations. Also noteworthy is that this decrease does not seem to have had a significant effect on F_{is}.

As shown in Table 9, the subdivision by year - 15 microsatellite loci (Tab 9a) reports results that are comparable to the analyses carried out by year - 10 microsatellite loci (Tab 9b). However, while for year - 15 microsatellite loci the reduction of H_e is significant ($P < 0.05$) for almost all years from 2002 to 2019, this is not the case of 10 loci used by De Barba *et al.*, 2010. This result indicates that 15 microsatellite loci give a more sensitive measure of a decrease in heterozygosity than a set of 10.

Table 9 Estimates of the main indices of genetic variability using subdivision by: (a) year, considering all 15 microsatellite loci; (b) year, considering the set of 10 loci used by De Barba *et al.*, 2010; (c) cohort; (d) generation; (e) subsampling groups. H_o, observed heterozigousity; H_e, expected heterozigousity; *A*, allelic richness; *number of private alleles*; F_{is}, inbreeding coefficient; Ne^{all}/Na, ratio between the average effective number of alleles per locus and average number of alleles per locus. The reported levels of significance (*P < 0.05, **P < 0.01, ***P < 0.001) were calculated through a paired t-test between the founders and every subpopulation for H_e, H_o, *A* and F_{is}.

(a)

pop	*Ho*	*He*	*A*	*n° private alleles*	*Fis*	*Ne^all/Na*
founder (9 ind.)	0.73	0.70	5.02	0.18	0.03	0.75
2002 (10 ind.)	0.74	0.68 *	4.79 *	0.02	-0.03	0.71
2003 (11 ind.)	0.73	0.67 **	4.49 **	0.00	-0.04	0.72
2004 (15 ind.)	0.75	0.67 *	4.25 **	0.00	-0.08*	0.71
2005 (18 ind.)	0.74	0.66 *	4.11 **	0.00	-0.09	0.71
2006 (22 ind.)	0.75	0.67	4.08 **	0.00	-0.10	0.74
2007 (23 ind.)	0.77	0.66 *	3.99 **	0.00	-0.16*	0.72
2008 (27 ind.)	0.74	0.65 *	3.90 **	0.00	-0.12	0.71
2009 (29 ind.)	0.74	0.64 *	3.88 ***	0.00	-0.13	0.70
2010 (36 ind.)	0.74	0.64 *	3.85 **	0.01	-0.15*	0.68
2011 (35 ind.)	0.73	0.65	4.09 **	0.19	-0.11	0.59
2012 (45 ind.)	0.74	0.65 *	3.99 **	0.13	-0.12	0.59
2013 (42 ind.)	0.74	0.65 *	3.86 **	0.00	-0.13	0.69
2014 (44 ind.)	0.73	0.65 *	3.88 **	0.00	-0.12	0.69
2015 (53 ind.)	0.71	0.65 *	3.88 **	0.01	-0.09	0.68
2016 (55 ind.)	0.72	0.66	3.91 **	0.01	-0.08	0.70
2017 (56 ind.)	0.71	0.65 *	3.88 **	0.01	-0.07	0.70
2018 (72 ind.)	0.70	0.65 *	3.80 **	0.00	-0.07	0.69
2019 (72 ind.)	0.70	0.64*	3.80 **	0.00	-0.08	0.71

(b)

pop	*Ho*	*He*	*A*	*n° private alleles*	*Fis*	*Ne^all/Na*
founder (9 ind.)	0.78	0.75	5.8	0.14	0.02	0.74
2002 (10 ind.)	0.79	0.74	5.57	0	-0.01	0.70
2003 (11 ind.)	0.79	0.73	5.11 *	0	-0.04	0.73
2004 (15 ind.)	0.81	0.73	4.82 **	0	-0.07*	0.71
2005 (18 ind.)	0.82	0.72	4.60 **	0	-0.11**	0.70
2006 (22 ind.)	0.83	0.73	4.59 *	0	-0.11*	0.76
2007 (23 ind.)	0.87	0.73	4.52 **	0	-0.17**	0.74
2008 (27 ind.)	0.84	0.73	4.43 **	0	-0.14*	0.74
2009 (29 ind.)	0.84	0.72	4.42 **	0	-0.15*	0.73
2010 (36 ind.)	0.85	0.72	4.38 **	0	-0.16*	0.72
2011 (35 ind.)	0.83	0.73	4.64 *	0.21	-0.13	0.62
2012 (45 ind.)	0.82	0.72	4.50 *	0.15	-0.12	0.62
2013 (42 ind.)	0.81	0.72	4.33 **	0	-0.13*	0.71
2014 (44 ind.)	0.81	0.72	4.38 **	0	-0.12	0.72
2015 (53 ind.)	0.79	0.71	4.34 **	0	-0.10	0.70
2016 (55 ind.)	0.79	0.71	4.35 **	0	-0.09	0.71
2017 (56 ind.)	0.78	0.71	4.31 **	0	-0.08	0.73
2018 (72 ind.)	0.77	0.71	4.24 **	0	-0.08	0.72
2019 (72 ind.)	0.77	0.71	4.21**	0	-0.08	0.73

(c)

pop	Ho	He	A	n° private alleles	Fis	Ne^{all}/Na
founder (9 ind.)	0.73	0.70	5.02	0.64	0.03	0.75
cohort1 (25 ind.)	0.75	0.67	4.1 **	0.04	-0.10	0.71
cohort2 (26 ind.)	0.72	0.62 **	3.68 ***	0.02	-0.15	0.70
cohort3 (51 ind.)	0.70	0.66	3.9 **	0.03	-0.06	0.70
cohort4 (37 ind.)	0.68	0.63 **	3.72 **	0	-0.08	0.69

(d)

pop	Ho	He	A	n° private alleles	Fis	Ne^{all}/Na
founder (9 ind.)	0.73	0.70	5.02	0.59	0.03	0.75
F1 (24 ind.)	0.75	0.67	4.07 **	0.03	-0.10 *	0.72
F1b (37 ind.)	0.74	0.62 **	3.68 ***	0	-0.18	0.70
F2 (11 ind.)	0.70	0.62 *	3.77 ***	0	-0.07	0.80
F2b (37 ind.)	0.69	0.63 **	3.73 **	0	-0.07	0.68
F3 (12 ind.)	0.67	0.61 **	3.68 **	0.04	-0.05	0.73

(e)

pop	Ho	He	A	n° private alleles	Fis	Ne^{all}/Na
founder (9 ind.)	0.73	0.70	5.02	0.34	0.03	0.75
group1 (17 ind.)	0.75	0.68 *	4.37 ***	0.02	-0.08*	0.69
group2 (30 ind.)	0.76	0.67	4.09 **	0.01	-0.11	0.71
group3 (30 ind.)	0.74	0.64 *	3.92 **	0.02	-0.13*	0.69
group4 (30 ind.)	0.72	0.65 *	3.90 **	0	-0.09	0.71
group5 (30 ind.)	0.72	0.66	3.99 **	0.02	-0.07	0.70
group6 (30 ind.)	0.68	0.64**	3.85 **	0	-0.05	0.69

The effective population size (N_e), or approximate number of reproducing individuals, calculated using both the LD and heterozigosity excess (H_e) methods, reported comparable but much lower values than the census number of individuals obtained with genetic monitoring (number of independent genotypes – N gen. rec., Tab 10) or from ecological methods based on photo/video-tracking and biologging combined with genetic monitoring (Groff *et al.*, 2020; N. nat. rec., Tab 10) for all database subdivisions used (except cohort, for which LD and H_e gave contrasting results). This underlines the importance of monitoring N_e over time, which is a more representative indicator of the reproductive potential of a population, contrary to what was thought until a few years ago, when the only parameter considered was the total number of individuals within a population (Willoughby *et al.*, 2015). The subsampling subdivision also gave comparable result to other subdivisions, indicating that this standard in conservation genetics gives reasonable estimates of N_e. However, in some cases the N_e and its confidence interval report 'infinite'; this is because the sampling error (that is *1/s*, with *s* representing the number of samples for a population) was subtracted from the initial N_e value. As a consequence, when the number of individuals sampled is low, the sampling error will be too high and, in these cases, the data obtained are not significant, so they cannot be considered (Waples *et al.* 2014).

Table 10 Population size indices for: (a) year - 15 microsatellite; (b) year - 10 microsatellites; (c) cohort; (d) generation; (e) subsampling groups. *N (gen. rec.)*, number of census individual through non-invasive genetic techniques; N_e, effective population size calculated from genetic data, based on both LD and Heterozigousity excess method. For the subdivision by years (a, b) the *N* calculated basing on ecological methods (*N (nat. rec.)*) is also reported for every year from 2002 to 2019.

(a)

pop	*N (nat. rec.)*	*N (gen. rec.)*	*Ne genetic data*			
			LD method		*H excess method*	
			Ne	*95% C.I.*	*Ne*	*95% C.I.*
founder	9	9	infinite	64.7 to infinite	infinite	11.5 to infinite
2002	10	10	28.2	12.4 to 2087.6	57.4	7 to infinite
2003	11	11	12.3	6.9 to 26.3	26.6	5.4 to infinite
2004	15	15	6.8	4.1 to 10.1	11.5	4.6 to infinite
2005	18	18	8.1	5.7 to 11.5	8.8	4.6 to infinite
2006	24	22	2.9	2.5 to 3.9	6.8	4.4 to 15.9
2007	24	23	3.3	2.8 to 5.2	4.5	3.4 to 6.6
2008	27-31	27	2.9	2.5 to 3.5	5.6	4.1 to 9
2009	25-30	29	4	3 to 5.7	5.2	4 to 7.6
2010	27-31	36	3.6	3.2 to 4.4	4.7	3.7 to 6.3
2011	33-36	35	4.8	3.7 to 6.6	6.3	4.7 to 9.8
2012	43-48	45	5.5	3.9 to 7	6.2	4.6 to 10
2013	40-49	42	5.9	4 to 7.7	5.3	4 to 8
2014	41-51	44	8.3	6.5 to 10.2	5.9	4.1 to 10.8
2015	48-54	53	7.3	5.8 to 8.9	8.5	4.8 to 49.8
2016	49-66	55	8.2	6.7 to 9.9	9.5	5.2 to 76.5
2017	52-63	56	7.8	6.3 to 9.5	11.7	5.7 to infinite
2018	60-78	72	9.3	7.8 to 11	11.7	5.8 to infinite
2019	82-93	72	11.3	9.4 to 13.4	8.4	5.7 to 16.6

(b)

pop	N (nat. rec.)	N (gen. rec.)	Ne genetic data			
			LD method		H excess method	
			Ne	95% C.I.	Ne	95% C.I.
founder	9	9	infinite	52.6 to infinite	infinite	9.5 to infinite
2002	10	10	31.8	11.4 to infinite	infinite	7.1 to infinite
2003	11	11	13.5	6.3 to 45.9	54.9	5 to infinite
2004	15	15	7.3	3.6 to 12.3	18.4	4.6 to infinite
2005	18	18	9.4	5.9 to 15.2	8.9	4.1 to infinite
2006	24	22	3.2	2.6 to 5.6	6.5	4.1 to 17.3
2007	24	23	5.2	3.1 to 7.7	4.2	3.2 to 6.3
2008	27-31	27	3.2	2.6 to 5.5	4.8	3.6 to 7.6
2009	25-30	29	5	3.1 to 7.2	4.6	3.5 to 6.9
2010	27-31	36	3.7	3.1 to 5.8	4.3	3.4 to 6.1
2011	33-36	35	5.5	3.7 to 7.7	5.6	4.1 to 9
2012	43-48	45	5.4	3.7 to 7.3	6.3	4.4 to 12
2013	40-49	42	5.4	3.7 to 7.7	5.6	4 to 9.8
2014	41-51	44	8	5.6 to 10.6	6.4	4.1 to 16.6
2015	48-54	53	7.8	5.6 to 10.6	7	4.4 to 19
2016	49-66	55	8.2	6.1 to 10.5	7.6	4.8 to 20.5
2017	52-63	56	8.4	6.2 to 10.7	9.5	5.4 to 58.4
2018	60-78	72	9.6	7.5 to 12	9.3	5.4 to 40.4
2019	82-93	72	11.5	9 to 14.5	8.8	5.4 to 27.4

(c)

pop	N (gen. rec.)	Ne genetic data			
		LD method		H excess method	
		Ne	95% C.I.	Ne	95% C.I.
founder	9	infinite	64.7 to infinite	infinite	11.5 to infinite
cohort1	25	8	6.1 to 10.4	6.6	4.5 to 13.5
cohort2	26	6.8	4.9 to 9.1	4.8	3.3 to 8.9
cohort3	51	12.8	10.5 to 15.5	16.3	6.2 to infinite
cohort4	37	24.4	18 to 34.4	8.2	5.3 to 18.7

(d)

pop	N (gen. rec.)	*Ne genetic data*			
		LD method		*H excess method*	
		Ne	*95% C.I.*	*Ne*	*95% C.I.*
founder	9	infinite	64.4 to infinite	infinite	11.5 to infinite
F1	24	2.3	2 to 2.6	6.2	3.9 to 17.5
F1b	37	9.2	7 to 11.9	4.7	3.1 to 11.4
F2	11	21.3	9.3 to 216.1	8.1	4.3 to infinite
F2b	37	22.8	17 to 31.6	8.9	5.3 to 32.3
F3	12	15.4	7.7 to 47.7	19	4.8 to infinite

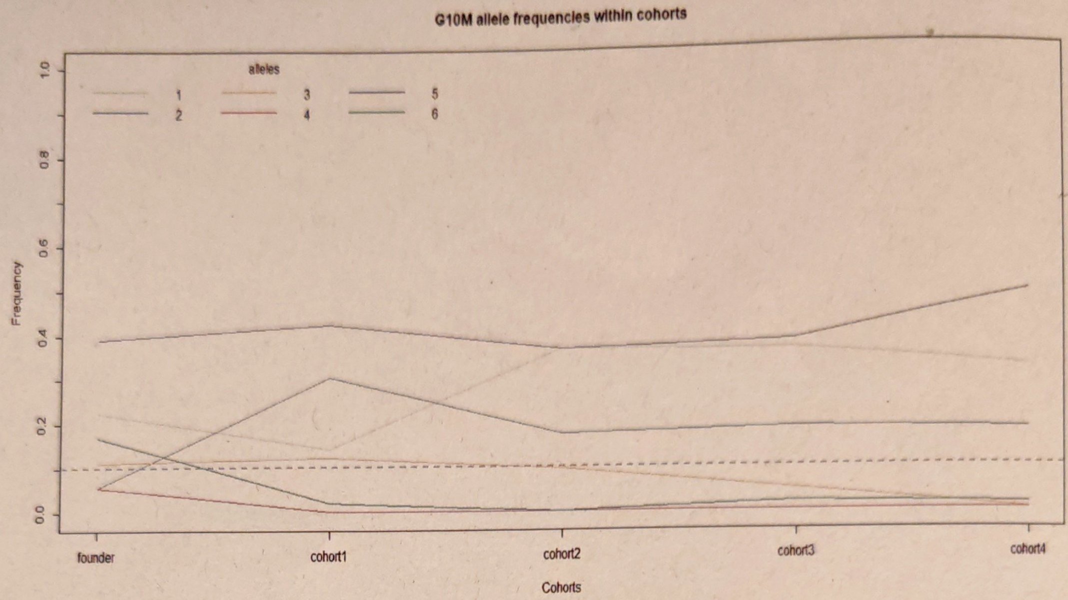
(e)

pop	N (gen. rec.)	*Ne genetic data*			
		LD method		*H excess method*	
		Ne	*95% C.I.*	*Ne*	*95% C.I.*
founder	9	infinite	64.7 to infinite	infinite	11.5 to infinite
group1	17	7.8	5.5 to 11	11.3	4.9 to infinite
group2	30	4.9	3.3 to 6.4	6.3	4.6 to 10.7
group3	30	3.9	3 to 5.6	6.8	4.1 to 22.7
group4	30	6.4	4.8 to 8.2	7.6	4.8 to 21.6
group5	30	14.2	11 to 18.6	10.3	5.4 to 466.7
group6	30	16.5	12.4 to 22.5	15.2	6.6 to infinite

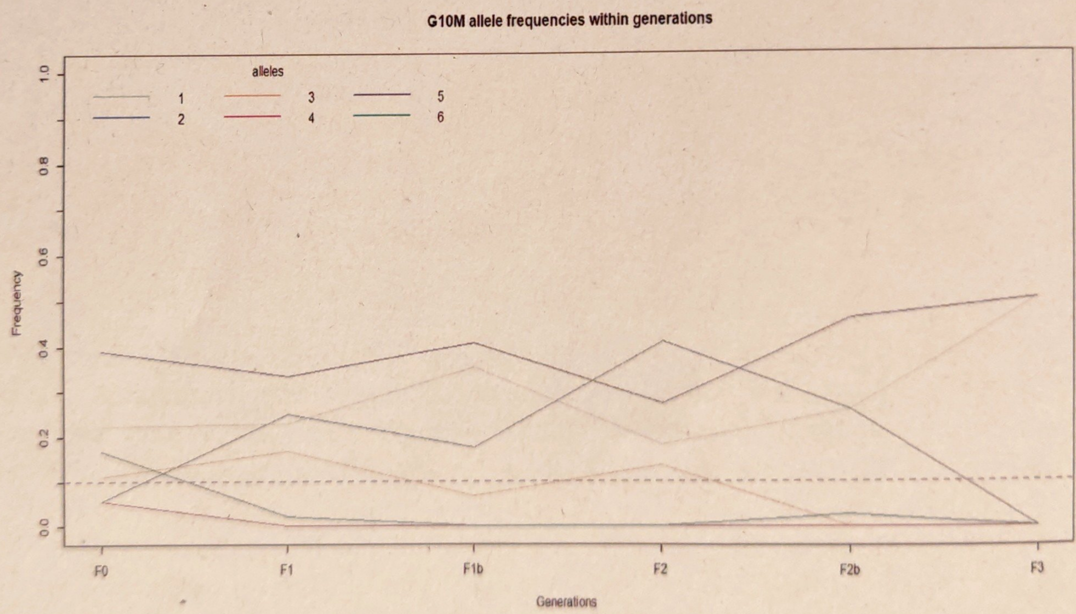
For the subdivision by years (Tab 10a, Tab 10b), the census number of individuals calculated using ecological techniques ('N nat. rec.') was comparable to N calculated using genotyping (number of independent genotypes, or 'N gen. rec.'), confirming that both methods provide useful estimate of total number of individuals in the population.
I also calculated the allele frequencies within all subpopulations, to assess whether there has been a significant reduction in the number of alleles due to a negative effect of genetic drift. Figure 10 shows the trend of the allele frequencies for 'G10M' locus over time as an example.

Figure 10 Allele frequencies for locus G10M within: (a) cohort; (b) generation; (c) subsampling

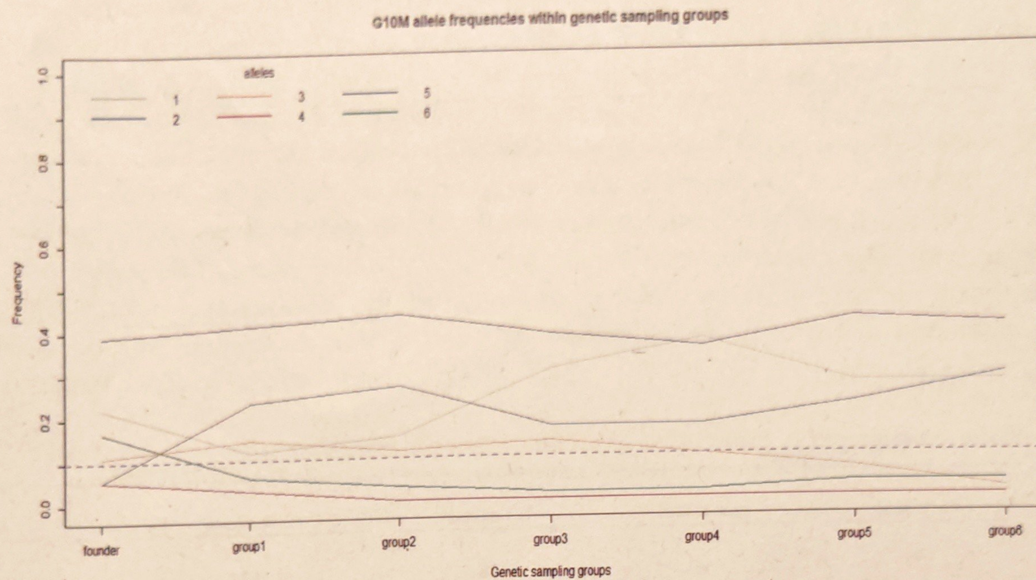
(a)



(b)



(c)



From the six starting alleles for this locus in the founder group, three (Fig 10a, Fig 10c) or four (Fig 10b) have been lost over time. In general, for all the subdivision criteria adopted, a marked loss of alleles from the founding individuals has emerged (Tab 11). Most of the lost alleles were initially rare (i.e. with a frequency lower than 10%), while new rare alleles appeared over time, so that frequencies generally decreased. This result was also expected, as it is well known that small and isolated populations tend to show a strong negative effect of genetic drift over time which leads to extreme values of allele frequencies, fixing some and leading to the loss of others (England et al., 2003; Honnay, 2013; Relethford, 2013; Templeton, 2019).

Table 11 Total loss of alleles from Founders to: (a) 2019, year – 15 microsatellite; (b) 2019, year – 10 microsatellite; (c) cohort4 (2017-2021); (d) generation F3; (e) group6 (2017-2019), subsampling.

(a)

FOUNDER		2019		
Tot alleles	Rare alleles	Tot alleles	Lost alleles	New rare alleles
77	16	66	11 (-14,28 % of the initial total)	11 (18,03% of the initial non-rare alleles)
			Initially rare	
			8	

(b)

FOUNDER		2019		
Tot alleles	Rare alleles	Tot alleles	Lost alleles	New rare alleles
58	14	48	10 (-17,24 % of the initial total)	7 (15,90% of the initial non-rare alleles)
			Initially rare	
			7	

(c)

FOUNDER		COHORT4 (born 2017-2021)		
Tot alleles	Rare alleles	Tot alleles	Lost alleles	New rare alleles
77	16	65	12 (-15.60% of the initial total)	3 (4.91% of the initial non-rare alleles)
			Initially rare	
			7	

(d)

FOUNDER		GENERATION F3		
Tot alleles	Rare alleles	Tot alleles	Lost alleles	New rare alleles
77	16	58	19 (-24.67 % of the initial total)	9 (14.75% of the initial non-rare alleles)
			Initially rare	
			10	

(e)

FOUNDER		GROUP 6 (alive 2017-2019)		
Tot alleles	Rare alleles	Tot alleles	Lost alleles	New rare alleles
77	16	66	11 (-14.28 % of the initial total)	11 (18,03% of the initial non-rare alleles)
			Initially rare	
			8	

Despite this, however, there was no significant genetic distance between the current population and founders for any of the criteria used, reporting a value of F_{st} between 0 and 0.02. For the interpretation of this result it is important to remember that the Wright fixation index (F_{st}) is calculated basing on the difference in heterozygosity between two populations (Relethford, 2013; Frankham *et al.*, 2006), which in this case study reported a non-significant decrease between the founders and the current population.

Finally, I simulated a scenario in which a single Slovenian male bear arrives in Trentino in 2019, to see the effects that this event would have on the total level of genetic variability of the population. Therefore, to the database, I added the genotype of a male bear of Slovenian origin sampled in Friuli Venezia Giulia Region, that is 'Gen-26'.

Table 12 shows that the addition of a single Slovenian male to the Trentino population would increase allelic richness (A) and the number of private alleles, although not significantly. H_o and H_e values would remain almost the same, as would the inbreeding level (F_{is}), because for the calculation of heterozygosity only non-rare alleles are considered, i.e. those with a frequency higher than 10%; therefore, the alleles brought by the new male, having a frequency lower than 10%, do not contribute to the calculation of such values. However, they contribute to the allelic richness and the number of private alleles, which turn out to be higher with the arrival of the new individual. The results of this simulation were encouraging and further simulations will be done to provide guidelines as to how many reproducing individuals would need to be added to restore the population to original levels of heterozygosity.

Table 12 Estimates of the main indices of genetic variability in: (a) 2019 using 15 microsatellites, compared with (b) 2019 using 15 microsatellites if a male from the Slovenian population arrived in Trentino

(a)

pop	*Ho*	*He*	*A*	*n° private alleles*	*Fis*
2015 (53 ind.)	0.71	0.65	3.88	0.01	-0.09
2016 (55 ind.)	0.72	0.66	3.91	0.01	-0.08
2017 (56 ind.)	0.71	0.65	3.88	0.01	-0.07
2018 (72 ind.)	0.70	0.65	3.80	0.00	-0.07
2019 (72 ind.)	0.70	0.64	3.800	0.00	-0.08

(b)

pop	*Ho*	*He*	*A*	*n° private alleles*	*Fis*
2015 (53 ind.)	0.71	0.65	3.88	0.01	-0.09
2016 (55 ind.)	0.72	0.66	3.91	0.01	-0.08
2017 (56 ind.)	0.71	0.65	3.88	0.01	-0.07
2018 (72 ind.)	0.70	0.65	3.80	0.00	-0.07
2019 (73 ind.)	0.70	0.65	3.87	0.03	-0.07

7 CONCLUSIONS

The study of changes in the level of genetic variability in reintroduced populations is of fundamental importance to assess the probability of their persistence in the long term and to apply appropriate management actions. This study provides an important example of the potential of non-invasive genetic techniques and its applications to monitor the genetic variability of a reintroduced population over time.

As expected from population genetic theory, in this small isolated population of large mammal, the levels of genetic variability, as estimated by standard indices, have decreased. Consequences of the founder effect were evident, especially with regard to the immediate reduction of allelic richness. Therefore, my initial hypothesis that the isolation of the population would have negative effects on its genetic variability was confirmed. In fact, my results reported a reduction in the number of alleles most likely due to drift acting more markedly on isolated

populations. However, contrary to what I expected, no negative consequences on inbreeding levels (F_{is}) were suggested. However, since the observed heterozygosity is currently decreasing and already significantly lower than the founder group, I expect the inbreeding coefficient to increase over time as well. This probability increases if other mating between relatives occur.
All the subdivision criteria used reported comparable results, so they all proved suitable for this type of study. In addition, the generation time of the brown bear, presumed to be about 5 years, was confirmed, since cohort and generation subdivisions reported the same trend of genetic variability.
The loss of genetic diversity was more significant when considering the 15 microsatellite loci currently used for individual identification compared to the 10 microsatellites used by De Barba *et al.*, 2010. This suggests that the 10 loci used in the previous study are not as sensitive to the trend of genetic variability within the population in the same way as the 15 microsatellites, underlining the importance of choosing an appropriate number of loci to be analyzed for studies of this type. The subdivision into genetic sampling groups also reported results that are comparable with all other subdivisions, thus confirming the suitability of this sampling method and standard for population size (i.e. 30 individuals) in conservation genetics studies.
Finally, the effective population size (N_e) calculated basing on genetic data was much lower than the census number of individuals based either on genotyping or ecological methods for each of the subdivisions, confirming the importance of considering N_e as a more accurate estimate of reproducing individuals. In this case study, according to Table 10a, N_e is 7-9 times lower than N. This presumably means that many adults are not actually reproductively active, which could ead to further decreases in heterozygosity and rare alleles.
For the future, regular genetic monitoring of the brown bear population in Trentino is recommended, in order to ensure accurate estimations of population parameters. For this type of study, newer, more sensitive markers could also be used, such as SNPs, which would give information about the effect of selection on the population and its evolutionary potential (Nielsen E. *et al.*, 2020). In fact, in recent weeks, a European consortium of conservation genetics laboratories is currently developping an SNP panel which will then be tested on low quantity/quality DNA from the Trentino population. Alternatively, HTS (High-throughput sequencing) could be used for microsatellite analysis (de Barba *et al.*, 2017). This technique provides direct access to microsatellites sequences through 'Illumina' technology. These authors report a more accurate individual identification and significant improvement of genotyping success (84%), even for low amounts of DNA as in the case of samples collected non-invasively; however, the costs of this technology could be prohibitive, especially for low numbers of samples, as is the case for the Trentino population.
Solutions are needed to maintain good levels of genetic variability in the future, for example through the exchange of additional individuals with the Slovenian bear population. In this way the total number of bears in Trentino would remain unchanged, but the gene flow would increase, albeit artificially, and consequently new alleles could be brought into the population, thus bringing genetic variability back to more acceptable levels and decreasing the risk of a possible inbreeding depression. In parallel, innovative methods for encouraging bear dispersal to

eastern Trentino and beyond to Slovenia, such as green corridors and bridges, are being considered by FEM and their collaborators.
Since the arrival of a single reproductive individual would increase, albeit slightly, two indices of genetic variability of the population, I will be conducting further simulations to estimate how many individuals carrying new alleles should be added to the population to increase the levels of heterozygosity to the initial values of the founding individuals.

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