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Challenging the loss of genetic variability in Italian brown bears (*Ursus arctos*): a genome-wide approach

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Background of the study species and populations

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Bear biology and ecology

Brown bears (*Ursus arctos*, Linnaeus 1758) are solitary animals. Relations between individuals are mainly based on mutual avoidance, except during the mating season (AA.VV. 2002). Much like the majority of other large carnivores, bears have wide home ranges and occur in low population densities. Females set up their home ranges next to their mothers' (philopatry), while males disperse (Clevenger & Purroy 1991). Female home ranges may overlap (Roth 1983). Bears are more active at dusk and night (Roth 1983). Their activity depends on environmental conditions, amount of food and human activity (Swenson et al 2000). In the Alps, brown bears mainly use forested habitat between 300 m and 1400 m a.s.l. (Mustoni 2004). Habitat use indicates that bears prefer deciduous and mixed forest, but areas with bushes and conifer trees are also regularly used (Preatoni et al 2005).

Brown bears are opportunistic omnivores. They typically collect food with the highest possible nutritional value available at any particular moment (AA.VV. 2002). The majority of food is of plant origin. Spring is the most challenging season, especially until the beginning of the growing season. Carcasses of animals that died during winter also represent an important spring food source (Clevenger & Purroy 1991, Frassoni 2002).

From the time the growing season begins until late autumn they like to graze and eat fruits of forest trees and plants (cornel, hazel, strawberries, blueberries, brambles)(Cicnjak et al 1987). In autumn, when they are accumulating fat for hibernation, forest trees (beechnut, acorn, chestnut, hazelnuts, walnuts) and fruit trees in orchards (pears, apples, plums) are important food sources. Bugs (ants, wasps, bees, wood beetles, chafers, weevil) and their pupae represent high-protein food sources for the bear (AA.VV. 2002, Clevenger & Purroy 1991).

On occasions bears hunt livestock, especially cattle. They also find food at open-air waste dumps. When they find food on people's properties (small cattle, orchards, bee houses…) and cause damage, conflicts with people arise.

The bear hibernates in winter; however, this is not deep winter hibernation as we know, for example, in dormice. Its body temperature only drops 2°C and its pulse and digestive system slow down (Swenson et al 1997). Since it does not drink fluids during this period, toxic waste starts accumulating, especially in urea. The bear's "winter hibernation" is actually a special form of starvation with the ability to neutralize toxic waste (Nelson et al 1973). Bears from southern European populations, such as the Italians, may be more active throughout the year. Spending winter in the den is probably an adaptation to lack of food during winter time and possibly to giving birth to cubs which are not fully able to thermoregulate (Swenson et al 2000).

Bears are known for their long lifespan, late sexual maturity, and extended reproductive cycle. It is a polygamous species. Mating takes place from mid-May to early July. After insemination embryos develop to the blastocyst stage. Further development is stopped until late November which is when implantation takes place. After that, gestation takes another 6 to 8 weeks. In January or February females give birth in the den to 1 to 4 cubs; they weigh approximately 0.5 kg (Hellgren 1998). Cubs are considered grown (juveniles) from when they are 1.4 to 2.4 years old. In the Scandinavian population, which is the most intensely researched European population, they discovered that the females give birth for the first time between ages 4 and 6. The interval between two gestation periods is relatively short, approximately 2.4 years (Swenson et al 2000).

Historical and current status and distribution

The brown bear is the most widespread terrestrial animal in the world, with a holarctic distribution in Europe, Asia, and North America, ranging from northern arctic tundra to dry desert habitats (Swenson et al 2000). Approximately 200,000 individuals are estimated for this species and therefore is considered at low danger of extinction from the IUCN (LR / lc) in its worldwide range, but many populations are extremely isolated and subject to a serious risk of local extinction.

During the prehistoric era, the species was distributed throughout the European continent, with exception of major islands such as Ireland, Iceland, Corsica and Sardinia (Servheen et al 1999; Ciucci & Boitani, 2000). The reduction of its range has increased in relation to human population growth and rapid transformation of natural habitat: deforestation, agricultural transformation of the territory, and direct persecution by hunting, led to a gradual fragmentation, isolation, and in some cases, the extinction of many carnivore wild populations (Randi 1993; Swenson et al 2000). The brown bear is one of the species more heavily affected by these processes: bears were exterminated from most of Europe Western Europe and many areas of Eastern Europe and Northern Europe (Swenson et al 1995, Breitenmoser, 1998). The species survived only in sparsely human-populated territories dominated by large forests, and where effective legal protection was established (Mustoni, 2004).

Today, twelve distinct and demographically isolated populations dwell in Europe. Only two are considered large populations (>5.000 individuals), four are medium populations size (500-5.000), one is a small population (100-500), while five have less than 100 individuals (Swenson et al 2000; Linnell et al 2002). The total consistency it is estimated at around 50.000 individuals, of whom only 1.000 are outside Russia. The populations with higher densities (100-200 bears/1.000 km2) are found in the Ukrainian regions and Romanian (Slobodyan, 1993, Ionescu, 1997), while those with minor densities are registered in Finland and Norway (0.5-1/1.000 km2) (AA.VV. 1996; Swenson et al 2000). Population densities are extremely variable due to differences in food availability, the rate of harvest by humans and the stage of population expansion/retreat.

Regarding Italy, a survey on historical distribution shows that, at least until the beginning of the 19th century, the brown bear was widespread throughout the Alps, including the Ligurian Alps (Bologna & Vigna Taglianti, 1985). The Alpine population was extended to the Eastern Alps towards the Dinaric mountain region in the Balkans. More south, the population was probably present from the northern Apennines to the Apuan Alps.

A second isolated population was dwelling in the central-southern Apennines, from the Sibillini Mountains to Campania and Puglia (Febbo & Pellegrini, 1990; Boscagli et al 1995).

Currently, the brown bear survives in three distinct geographic areas in Italy, the first is in the central Alps, with the core area located in the Trentino region; the second is situated in Apennine mountain range of central Italy, with the core area located in the Abruzzo, Lazio and Molise National Park. The third area of presence is in eastern Italy, in the region Friuli-Venezia Giulia, where is located not a reproductive population, but only a few individual males dispersing from the Dinaric population of Slovenia, are annually present. (Krofel et al 2010).

The Alpine population (*Ursus arctos arctos*)

In the 17th century, the brown bear was still considered widely distributed in Northern Italy. The fast process of numerical decline and distribution contraction in the Alps began during the 18th century due to direct persecution and indirect human-induced causes. Historical data show a true extermination, for instances, between 1861 and 1879, 226 brown bears were killed in Trentino-Alto Adige; and 58 in the province of Sondrio (Castelli, 1935). The species was legally protected in 1939 (Testo Unico, 5 June 1939, n. 1016), but its decline continued due to illegal harvest. The resulting numerical decline led to a contraction of range distribution in both central and eastern Alps. As a result, the Alpine population disrupted into two small disjoint groups (Dupré et al 2000), and the species became isolated in the central Italian Alps. In the eastern Alps the last specimen in the region was killed in 1911. In 1990, bear presence in the entire Alpine range appeared stable, but ascertained with confidence only within the Brenta mountain group, in Trentino-Alto Adige (Dupré et al 2000). The last reproduction event was recorded in 1989 and the species survived the anthropic pressure until 1997 when the species was defined “biologically extinct”, due to the presence of only 1-3 relict individuals of the former large Alpine-Balkan population (Dupré et al 2000).

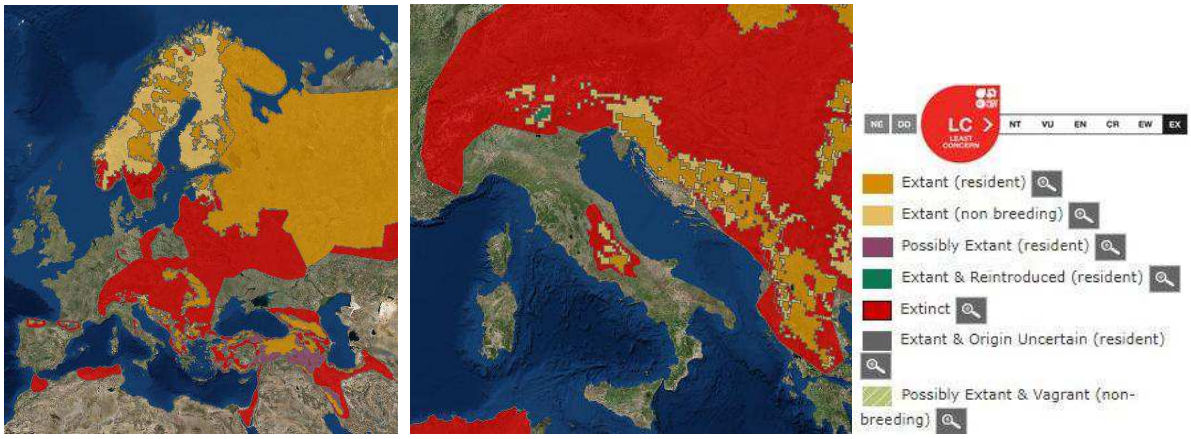
In 1999, a translocation plan was initiated by the Parco Naturale Adamello Brenta (PNAB) in collaboration with the Trento province and the Italian Wildlife Institute (INFS, now in ISPRA, the Italian Institute for Environmental Protection and Research). The program was co-funded by the European Union through the “LIFE Ursus” program. Between 1999 and 2002, 9 bears were captured in Slovenia, fitted with radio-collars and released into the Park. The

use of the Slovenian population as the source for augmentation was justified by historic and genetic considerations (Dupré et al 2000; Randi et al 1994). Following the translocation, local managers were facing many challenges related to monitoring of the reintroduced population and needed critical data on reproduction and demography to evaluate the success of the reintroduction. Obtaining this information by physically re-capturing and handling individuals was not considered feasible for the small population and noninvasive genetic sampling (NGS) was preferred over traditional field-based methods (De Barba et al 2010). The genetic monitoring program started in 2002 in order to follow its demographic and geographical expansion and changes in the genetic composition. First results were reported by De Barba et al (2010) and were promising: the population expanded its distribution, increased rapidly, and the genetic diversity was high (expected heterozygosity He=0.74-0.79; allelic richness Na=4.55-5.41). However, even if the monitoring project is still ongoing, data on population demographic trends, reproductive success, and population genetics dates back to 2002-2008, and a comprehensive study on population dynamics after 15 years from the reintroduction program is still lacking. Nevertheless, preliminary data showed that the population was still growing at the end of 2012 (growth rate: 15.6%) and it was further expanding its distribution area through the Italian Alps and abroad (Tosi et al. 2015).

Together with the monitoring project in the central Alps, the Autonomous Region of Friuli-Venezia Giulia, in collaboration with the University of Udine and ISPRA, started to collect non-invasive samples to monitor bear occurrence in Eastern Italy. The first sporadic reports of bear presence in Friuli-Venezia Giulia date back to the second half of the 60s (Dupre et al 2000), but the recovery of the numerical consistency of the Dinaric bear population in Croatia and Slovenia led to its progressive expansion to the north-west, with consequent recolonization of Italian and Austrian Alps. The monitoring project started in 2001 but comprehensive and updated data on bear occurrence in this areas have not been publishedThe Appennine population (*Ursus arctos marsicanus*)

The Apennine brown bear distribution has progressively been reduced since the 17th century (Febbo & Pellegrini 1990, Boscagli et al 1995), but the largest range reduction occurred over the past 200 years, mostly due to direct human persecution (Febbo and Pellegrini 1990). By the 1970s the remnant bear population survived almost exclusively in the Abruzzo, Lazio and Molise National Park (Zunino & Herrero 1972). Many attempts to make formal assessments of the population abundance were carried out between the 1970s and 1980s using different signs of presence, approaches and methods, but a reliable estimate of the Appenine brown bear population size has never been produced. The first estimate of 43 bears (95% CI:35-67) was produced applying a capture-mark-recapture modeling to DNA data collected during 2004, according to a systematic sampling design (Ciucci & Boitani 2008). A second estimate was conducted in 2015: 51 bears were estimated (95% CI = 47–66), including cubs. Comparing results with the survey in 2008, it is clear that the Apennine brown bear population is small, but has not been declining in recent years. Additionally, the relatively high (closure corrected) density (39.7 bears/1,000 km2; 95% CI = 36.6–51.4) indicated that habitat productivity within the core range was adequate for bears and that effective conservation of this small bear population should have aimed to expand the bears' range across a larger portion of the central Apennines (Ciucci et al 2015).

The current extent of occurrence of the population appears to be differentiated into a core area inside the Park and some peripheral areas, where a limited number of dispersing bears are irregularly detected at much lower densities. The peripheral area includes a larger network of protected areas in the central Apennines. A recent study examined the bear occurrence outside the core area using different sources of data (telemetry relocations, scats and DNA-verified hair samples, sightings, indirect signs of presence, photos from camera traps, and damage to properties). Despite stable occupancy by adults outside the core area, reproducing females appeared to be restricted to the core portion of the range (Ciucci et al 2017). The peripheral area is extended northwest to the Lazio Ragion, northeast and east of the core area and is partly included within a larger network of protected areas in the central Apennines (Monti Simbruini Regional Park, Monte Genzana Alto Gizio Natural Reserve, Majella National Park, Gran Sasso Monti della Laga National Park).



1.2 – EUROPEAN AND ITALIAN BEAR DISTRIBUTION

Major threats

As wide-ranging omnivores, brown bears are attracted into areas with available human-related foods. Areas of high human use that attract bears may serve as significant mortality sinks (Nielsen et al 2004, 2006). Additionally, bears living near humans may be killed inadvertently (e.g., vehicle or train collisions), poached, or killed by people hunting for other species. In addition to direct removal of brown bears, many other human activities (such as agriculture, plantation forestry, highways, hydroelectric and wind power developments, and human settlements) eliminate, fragment, or erode the value of bear habitat (Proctor et al 2005, Waller and Servheen 2005, Proctor et al 2012). Habitat fragmentation is a serious threat that isolates population units with deleterious demographic and genetic impacts (Proctor et al 2005, 2012).

The key threats to bears in Europe are: habitat loss due to infrastructure development, disturbance, low acceptance, poor management structures, intrinsic factors, accidental mortality, and persecution. Most threats are expected to increase in the future (Kaczensky et al 2013).

Phylogenetics of brown bear in Europe

Studies on the genetic variability of the brown bear across its distribution have been conducted using mitochondrial DNA analyses (Hartl & Hell 1994; Kohn et al 1995; Miller et al 2006; Randi et al 1994; Taberlet & Bouvet 1992, 1994; Talbot & Shields 1996; Waits et al 1998). All bears in Europe belong to the same subspecies (*Ursus arctos arctos*). However, these studies revealed the presence of two highly divergent mitochondrial DNA lineages in Europe differing by the 7.13% of the control region sequence (Randi et al 1994; Taberlet &Bouvet 1994): the Eastern lineage (Romania, Slovacchia, Russia, Finland, Estonia, Sweden), and the Western lineage, which is further subdivided into the Northwestern lineage (Norway, Sweden and Pyrenees) and the Southwestern lineage (Romania, Bulgaria, Greece, Balkans, Italy) showing lower degree of divergence. During the Holocene, the bears expanded following forestation and deglaciation, starting from the refugia where were confined. Today the geographical separation between the Eastern and the Western lineage is quite clear. The two lineages are sympatric only in Romania (Kohn & Knauer 1998, Zachos et al 2008), while a contact zone between the Eastern and Northwestern lineage is found in Sweden. These data were corroborated using nuclear data (Tammeleht et al 2010).

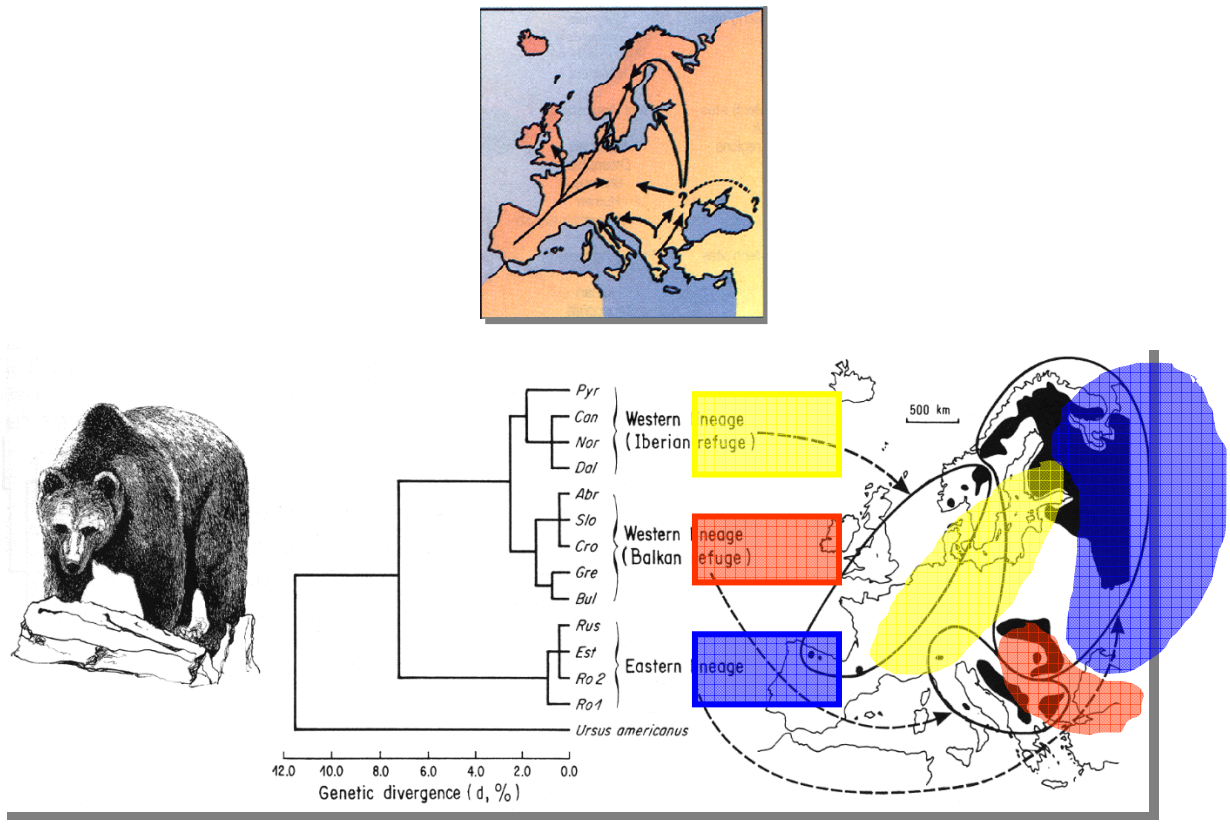
The three evolutionary lineages suggest the presence of three glacial refugia during Pleistocene. Taberlet et al (1998) hypothesized three glacial refugia in Europe, the Iberian, the Italian, and the Balkan. Saarma et al (2007) studied the mtDNA structure of bears from Eastern Europe and suggested that Carpathians formed glacial refugium for bears of the eastern lineage. Recently, more complex scenarios are emerging, with a proposed model of expansion and contraction to southern refugia in response to Quaternary climatic fluctuations and geneflow among populations during the last glacial maximum (Valdiosera et al 2007). Another hypothesis suggests that bears were not all restricted in glacial refugia, but survived in the cold tundra-steppe of central Europe and recolonized central Europe after the glacial maximum. The reasons for the detection of isolation of the European populations during the Pleistocene glaciations using mtDNA is probably due to the strong philopatry of females (Støen et al 2006) and severe reduction in population size and distribution (Zedrosser et al 2001).

These lineages correspond to three separated geographic areas and, since evolve separately, are considered distinct Evolutionary Significant Units (ESU) (Randi et al 1994; Taberlet & Bouvet 1994). Each of them significantly contributes to the genetic diversity of the species. This aspect must be taken into consideration for reintroduction and restocking projects. Each of these units should be preserved taking into account the genetic structure of the species. Translocations of reproducers inside of the same ESU preserve the genetic diversity and structure of the species, while gene flow between different ESU should be avoided.

The mitochondrial DNA confirmed that bears from the historical core area in the central Alps share the same mtDNA haplotype with the Croatian bears (Randi et al 1994). Therefore, bears present in the Balkan Peninsula and those surviving in the central Alps belong to the same phylogeographic unit (the Southwestern line). According to this, bears from Slovenia were translocated in Trentino in order to repopulate central Italian Alps.

The phylogenetic history of the Apennine population is different: historical information (Febbo & Pellegrini 1990) and mtDNA characteristics (Randi et al 1994) indicate that the Apennine population has been isolated from the Alpine population for at least 400-600 years (Randi et al 1994, Lorenzini et al 2004). The persistent isolation and population bottlenecks, caused by human persecution and habitat loss and fragmentation, led to the stochastic depletion of its original genetic diversity and (Lorenzini et al 2004). Consequently, the Apennine population shows one of the lowest levels of genetic variation in the world today (Skrbinšek et al 2012).

The mtDNA characteristics of this population corroborate the hypothesis of an ancient isolation of the Apennine population from the larger Alpine-Dinaric population: its mitochondrial haplotype is slightly divergent from that of the Alpine-Balkan populations (Randi et al 2007). A study on cranial morphological measurements clearly indicated that the Apennine bear is morphologically distinct from both a western (Alps, Pyrenees, Balkans, and Rhodopi) and an eastern contingent (Caucasus and Transcaucasus), therefore suggesting that the Apennine population should be considered as a separate taxon (Loy et al 2008). Thus, genetic and morphological data concur that the Apennine population should be considered as a separate taxon, namely Ursus arctos marsicanus, endemic of central Italy.



1. 2 - EUROPEAN PHYLOGENETIC LINEAGES AND HYPOTETICAL ROUTES FROM GLACIAL REFUGIA

The framework: recent and ongoing conservation measures

Many conservation measures have been taken in order to protect this species in Italy and in the neighboring countries. Specific European financial programs, such as LIFE-projects, Horizon2020 and Interreg projects, exist to help European member countries to implement conservation plans for protected species.
This PhD thesis is part of the following completed European programs for protection of brown bears in Italy:

- **LIFE Ursus - LIFE96 NAT/IT/003152: "Protection of Brenta brown bear population"**

Main goals the reintroduction program in the Brenta mountain region of central Italian Alps were: a) to translocate brown bears from Slovenia and avoid their extinction in the Alps;to assure the continuity of brown bear presence in the region and preserve the legacy of the native brown bear population; to re-establish a minimum viable population of 40-60 bears in the central Alps in 20-40 years and a brown bear metapopulation in the Alps in the longer term; b) to promote the coexistence between humans and bears by increasing awareness of the human population towards brown bears and the re-introduction project, through environmental education and media information, and involving local stakeholders in the project; c) to mitigate human-bear conflicts through protocols for damage evaluation, establishment of damage compensation schemes, prevention of damage to properties, and management of problem bears and of emergency situations; d) to plan monitoring and scientific research to measure success of the reintroduction and allow timely intervention if deemed necessary; e) to establish a network, at the national and international level, between the different relevant authorities to promote population level management.
A feasibility study was carried out by the former National Wildlife Institute (now ISPRA) to evaluate the environmental, administrative, socio-economic and normative aspects of a brown bear reintroduction in the central Alps in Italy based on the analysis of ecological, social and economic data. Based on a sample area of 6,495 km^2, the study verified the existence of a minimum suitable habitat of ~1,700 km^2 for supporting a minimum viable population of 35-50 bears, taking into account bear ecological requirements, environmental features, and human presence. The attitude of the human population living in non-urban areas was surveyed and found to be mostly (>70%) favorable to the re-introduction, despite lower levels of acceptance in some areas. The study also highlighted the necessity of improving prevention and compensation measures for damages possibly caused by bears. Finally, it was determined that, to achieve project objectives 9 bears (3 males and 6 females and approximately (2-6 years old) should be released in the area were the last relict bears of Trentino still existed. The Slovenian population should be the source of the translocated bears given the short time the populations have been separated, the behavioral characteristics, and the sustainability of the removal (stock taken from the Slovenian hunting quota). The study concluded that a re-introduction was feasible and could lead in the mid- to long-term to the successful re-establishment of the species in the central Alps.
Translocations took place during four years, between 1999 and 2002. Bears were captured in two hunting reserves in southern Slovenia and released in the Parco Naturale Adamello Brenta in the western part of Trentino. An additional female was released to replace one that died in an avalanche shortly after release. All re-introduced bears were equipped with a VHF collar and two ear tags to allow precise determination of their position, at least twice per day, and evaluate potential risks to people and properties, therefore preventing situations of possible conflicts with humans. Radio-tracking was the main monitoring method from 1999 to 2003 and provided important data on survival, habitat use, and distribution of the translocated bears. Radio-tracking through GPS/VHF technology is still used for close monitoring of problem bears. Starting in 2003, genetic monitoring became the principal mean to obtain demographic, reproductive, ecological, distribution and genetic information on the released bears and their descendants. The method is based on the analysis of the DNA extracted from biological samples, mostly bear hair and feces collected non-invasively in the field, using a variety of sampling techniques, but occasionally also tissue, blood, and bones retrieved during capture operations or from bear carcasses. Data from radio and genetic monitoring were complemented with additional information from traditional sign survey, visual observation (i.e. female with cubs), and camera traps.

- **LIFE ARCTOS - LIFE09 NAT/IT/000160: "Brown Bear Conservation: Coordinated Actions in the Alpine and Apennine Range"**

  This project was the natural continuation of the previous LIFE project, aiming at promoting the conservation of the brown bear populations not only of the Alps, but also of the Apennines, sustaining their recovery by means of management measures, consistent with the plantigrade presence, as well as the reduction of conflicts with the anthropic activities. In addition, it promoted information and awareness among the main stakeholders.

  The project aimed to achieve the following objectives: a) make livestock husbandry practices and regulations more compatible with the needs of bears; b) develop, promote and implement best practices for monitoring and controlling livestock diseases that are potentially transmittable to the bear population; c) increase the number of farms in the bear's range adopting effective prevention measures and increase by at least 30% the use of compatible husbandry techniques; d) facilitate the involvement and participation of the social sector; e) encourage administrations to adopt the tools, best practices and guidelines developed; f) significantly increase information and awareness in local communities in areas where bears are present; g) implement emergency teams in the bear areas in the Alps and Apennines.

  The ARCTOS project has implemented a series of actions referring to four line activities aimed at mitigating the threats concerning the brown bear: raising human practices more compatible with the presence of brown bear with particular attention to breeding of livestock; reduce the bear-human hostilities derived mainly from damage caused by the plantigrade to farms, by their frequentation of human settlements and habit to nourish with organic garbage.

  Support has been provided also to farms located in territories with bear presence, both in the Alps and the Apennines, consisting of electric fences transferred on free loan for use. Bins for organic waste, provided with anti-bear lids, were placed in the critical areas in order to dissuade animals from getting close to the human settlements; through the use of barriers and road signs the vehicular traffic has been limited in the areas frequented by bears; manage of bear feeding sources, especially buckthorns (*Rhamnus alpinus*) in the Apennines. These shrubs are very appreciated by bears, especially in critical periods, but threatened by wood encroachment and leaf grazing by wild and domestic ungulate. In order to conserve the buckthorns and to ensure bears accessibility to such an important source of food, thinning of beech woodlands, coppicing of dead buckthorns and planting of thousands of seedlings were performed; estimates of the brown bear populations were performed. Although the populations in Italy are small sized, no scientific information and data on their effective size were available for the Apennines and for expansion areas in the Alps. A standard protocol for collecting, analyzing and storing genetic data on Italian bears was elaborated.

  Other two ongoing projects have the aim of protecting the Alpine brown bear population, with a particular interest in expanding the environmental connectivity and thus the distribution of this population through the Alps:

- **LIFE DINALPS LIFE13 NAT/SI/000550: "Population level management and conservation of brown bears in northern Dinaric Mountains and the Alps"**

  It's the first project aiming at encouraging the natural expansion of brown bear from the Dinaric Mountains into the Alps. Other project's main objective focuses on establishing a more strategic territorial approach to the conservation, management, and monitoring of brown bear populations in a human-dominated, politically and physically fragmented landscape of northern Dinaric Mts. and the Alps. The project area extends through four countries: Slovenia, Croatia, Italy, and Austria.

  The main objective of the project is providing a population-level monitoring, management and conservation of brown bears in northern Dinaric Mountains and south-eastern Alps to overcome the current local-scale practices of brown bear management, establishing a tightly-knit transboundary network of professionals involved in these issues, optimize monitoring methods and their application, start with long-term transboundary monitoring, and provide first baseline data about these bears at the large-scale, transboundary level.

  Other objectives are decreasing of human-bear conflicts and promotion of coexistence through a variety of actions, from finding solutions in preventing bears from reaching anthropogenic food, to explore carrion from game road kills as an alternative natural source of protein, promoting bears as an eco-tourist attraction, exploring public attitudes towards bears, and using this for targeted educational and promotional activities to enhance understanding of this species and promoting co-existence.

  Lastly, the natural expansion of Brown bear from Dinaric Mountains into the Alps is promoted, since habitat modeling has shown that the Alps are capable of supporting a bear population and the small reintroduced population in Trentino is thriving and natural expansion is slow. A multidisciplinary approach is used to look into this issue and try to understand the social and physical barriers to expansion and the corridors that need to be protected. These

informations are used to provide solutions to slow down further habitat fragmentation, increase acceptability of bears in the areas where they are currently not permanently present, but where is the expansion is expected to occur.

- **BearConnect: "Functional connectivity and ecological sustainability of European ecological networks – a case study with the brown bear"**

  The BearConnect project aims at: evaluating functional connectivity and factors influencing brown bear distribution, movements, and effective dispersal in current and future landscapes scenarios, understanding the role brown bears have in ecosystems, with focus on trophic interactions and associated ecosystem services, assessing the effectiveness of existing ecological networks for supporting the resilience of brown bear populations and associated ecosystem services, providing spatially explicit guidelines for the improvement of ecological networks to be used in landscape connectivity planning for the conservation of brown bears and other species in Europe.

  In order to accomplish and achieve these objectives, the BearConnect project promotes further actions: coordinate across Europe to combine different data types available for the 10 European brown bear populations; analyse telemetry, demographic, genetic and ecological data to evaluate patterns of functional connectivity and landscape effects on bear movement and gene flow, derive the structure of food web interactions and the economic value of a key ecosystem service provided by brown bears; predict future changes in range dynamics of the brown bear and its food resources; use quantitative models and simulations to assess whether existing ecological networks are suitable for conserving biodiversity and ecosystems functions and where management actions are required for improvement.

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Introduction to conservation genetics

Conservation genetics applied to small populations

Conservation is about preventing species from going extinct and to help them persist in the future. However, conservation efforts that aim to sustain a certain species in the long-term require knowledge not only about the number of individuals in a population but also about the genetic characteristics: this is the conservation genetics's area of interest**.** Conservation genetics is derived from population genetics, a discipline that describes the genetic composition of populations to understand the causes that determine changes (evolutionary forces). Every species is made up of many evolutionary units, the populations, that contain a certain quantity of genetic variability on which evolution can act. Genetic variability in populations is described through allele frequencies that can vary in the course of generations due to mutations, natural selection, migration or genetic drift.
Conservation genetics uses population genetic theory and techniques to reduce the risk of extinction in threatened species. Its longer-term goal is to preserve species as dynamic entities capable of coping with environmental change. The field of conservation genetics also includes the use of molecular genetic analyses to elucidate aspects of species' biology relevant to conservation management.

Major issues in conservation genetics applied to small populations include (Frankham et al 2002, modified):

- Use of molecular markers for demographic monitoring of populations (minimum number of individuals, effective population size, population estimates)
- The deleterious effects of inbreeding on reproduction and survival (inbreeding depression)
- Deleterious effects on fitness that sometimes occur as a result of outcrossing (outbreeding
- depression).
- The loss of genetic diversity and ability to evolve in response to environmental change (loss of evolutionary potential)
- Fragmentation of populations and reduction in gene flow
- Random processes (genetic drift) overriding natural selection as the main evolutionary process
- Accumulation and loss (purging) of deleterious mutations
- Genetic management of small captive populations and the adverse effect of adaptation to the captive environment on reintroduction success
- Definition of management units within species
- Use of molecular genetic analyses to understand aspects of species biology important for conservation (mating system, dispersal and migration patterns, behavior)

A central issue in conservation genetics is the level of genetic variation present, a prerequisite for evolution (Pertoldi et al 2007; Väli et al 2008). The rates of adaptive evolution need to, at least, match the rate of environmental change in order for a population to persist (Pertoldi et al 2007). Two potential consequences may be envisioned for loss of genetic variability: a) low genetic variability can be a threat in the long-term for adapting and evolving in disturbed habitats and under changing environmental conditions; and (b) inbreeding may occur in small, fragmented and isolated populations (i.e. increased relatedness and homozygosity between individuals), posing an immediate threat to fitness in such a population (Pertoldi et al 2007; Väli et al 2008). An important prerequisite for the design of conservation strategies is information on the speed at which populations become inbred (Pertoldi et al 2007). A common rescue-strategy adopted by conservation genetics includes the increase of gene flow among populations for the maintenance of genetic diversity and alleviating inbreeding depression (Pertoldi et al 2007). However, high levels of gene flow can reduce the capacity of populations to stay adapted to local conditions or introduce mal-adapted genes that can reduce the viability of populations, known as outbreeding depression (Pertoldi et al 2007).
A further important issue in conservation genetics is the current structure as well as a history of a population or species, both in a demographic and phylogenetic sense (Pertoldi et al 2007). Evaluation of levels of genetic diversity is therefore common in population genetics and is particularly important in conservation genetics (Väli et al 2008). Comprehensive management plans for any species of conservation concern should include plans for maintaining existing genetic diversity, both to ensure ability to adapt to changing environments and to preserve the possibility of future speciation (Lacy, 1997).

As mentioned above, European brown bear populations vary greatly in size, from large populations near Russiam Carpathian mountains and the Dinaric-Pindos area, Balkan peninsula, and Scandinavia, to extremely small and isolated populations in the Cantabrian mountains, the Pyrenees the Apennine mountains and the Alps (Zedrosser et al 2001). Nuclear DNA diversity has been investigated in many of these populations using microsatellite markers. Results clearly showed that low genetic diversity occured in the smallest populations. The small population may suffer from genetic drift and inbreeding (Taberlet et al 1997, Lorenzini et al 2004, Pérez 2009). Inbreeding depression has not been documented in wild brown bear populations, but captive brown bears in Nordic zoos show the reduction in litter size and increased incidence of albinism due to inbreeding (Laikre et al 1996).

Genetics markers: from microsatellites to SNPs

A gene or DNA sequence, present with two or more variant of the same nucleotide sequence, is defined as polymorphic and can be used as genetic markers. Genetic markers are the main tools used to study the genetic variability within and among populations, in fact, they allow to estimate which alleles are present (Sunnucks, 2000). A genetic marker can be represented by any variable and hereditable characteristics in populations, and it always determined by genes, and not by the environment. The main characteristics of a molecular marker are polymorphism, expression stability during environmental, ontogeny and morphologic changes, well identifiable and amplifiable, Mendelian heredity, expression codominance.

The genome of vertebrates and many other living organisms is largely made up of coding and non-coding DNA sequences. The first ones are organized in functional domains and are necessary to regulate the protein synthesis consisting of a first phase of transcription of DNA into messenger RNA followed by a phase of translation of the messenger RNA into protein. On the contrary, the second ones exist in families of repeated sequences, also called VNTRs (variable number tandem repeats).

Many different types of genetic markers can be used for genetic conservation applications: restriction enzymes and restriction fragments length polymorphisms analysis (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP) and some kind of VNTRs (satellites and minisatellites) were extensively used in the past but are now obsolete.

More efficient markers took their place in the recent years: in the last decades STRs, also known as microsatellites, have become the most widely used DNA marker in conservation genetics: they are a classified as VNTRs, non-coding regions characterized by tandemly repeated sequences, present in many thousands throughout the entire eukaryotic genomes. STRs are codominant and made up of very short repeats, from 2 to 8 nucleotides, repeated only a few times, that produce clusters of a few dozen or few hundred nucleotides per locus. The microsatellite mutation results in a change in the number of repeats. The main advantage of STRs is that they are usually highly polymorphic, even in small populations. The high polymorphism result from a high mutation rate (10^{-04}-10^{-05} substitutions by replication per locus), primarily due to slippage during replication. This characteristic makes them particularly suitable to identify the unique multilocus profile and sex of the sampled individuals (*fingerprinting* analysis). Microsatellites are usually amplified using PCR and alleles are visualized using gel electrophoresis to separate fragments on the basis of differences in length resulting from different numbers of tandem repeats. The first step using SNPs is to identify single nucleotides that are polymorphic using a discovery panel of a small number of individuals. This is commonly done through DNA sequencing and once SNPs have been discovered, many tecniques exist among SNP genotyping.

The composition of microsatellite sequences is variable, although there are many poliA/poliT regions among vertebrates (eg AAAAAAAAAAA or TTTTTTTTT), which can not be used as genetic markers because extremely unstable during polymerase chain reaction. The CA/GT motifs, on the other hand, are the sequences of the most common dinucleotides. There are also repeated sequences of trinucleotides (eg CAG or AAT) or tetranucleotides (eg CAGT or AATG).

Today microsatellites are widely used in population genetics and are able to reconstruct the structure and history of populations, parental relationships between individuals and to determine the main population genetic parameters.

Recently, single nucleotide polymorphisms (SNPs) are becoming the marker of choice in evolutionary and conservation genetics, as next-generation sequencing techniques are developing and genomic sequence information accumulates. SNPs are abundant and widespread in coding and non-coding regions of many species' genomes (Brumfield et al 2003, Morin et al 2004). These polymorphisms are base substitutions, insertions, or deletions that occur at single positions in the genome (Budowle, 2004). The least frequent allele should have a frequency of 1% or greater to be considered as an SNP. Because the mutation rate at single base pair is low (about 10-8 changes per

nucleotide per generation). SNPs usually consist of only two alleles, thus are typically bi-allelic markers. SNPs in most species tend to be transitions, this because transversions in coding regions are more likely to cause an amino-acid substitution than transitions and be subject to selection.

As a biallelic marker, SNPs are innately less variable than microsatellites but are the most prevalent form of genetic variation and hence there is a substantial increase in the number of loci available (Brumfield et al 2003). Furthermore, the simpler mutational dynamics of SNPs lends the advantage of a lowered rate of homoplasy, and, importantly, there is a capacity for rapid, large-scale and cost-effective genotyping (Chen & Sullivan 2003; Schlötterer, 2004). Another advantage of SNPs is that it is much easier to standardize the scoring of genotypes when more than one laboratory is studying the same species. Moreover, SNPs are especially useful for studies involving partially degraded DNA (as non-invasive and ancient samples) because they are short and thus can be PCR-amplified from DNA fragments of less than 50 bases.

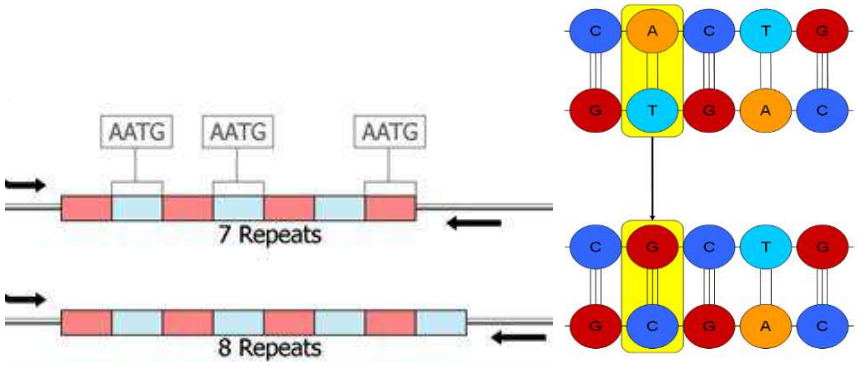

FIG 2. 1 - SCHEMATIC REPRESENTATION OF A STR(ON THE LEFT) AND SNP (ON THE RIGHT) POLYMORPHIC LOCUS

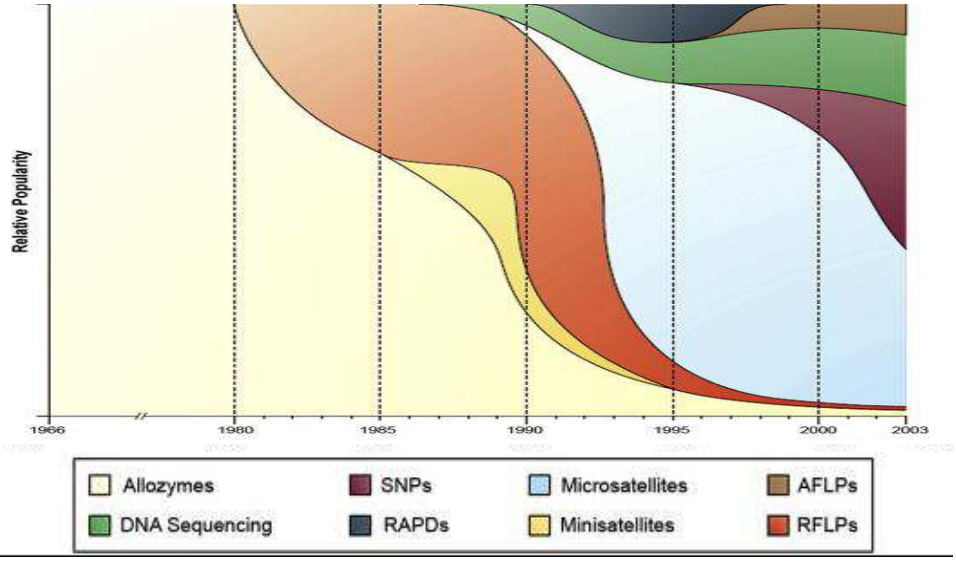

FIG 2. 2 - CHANGING RELATIVE POPULARITY OF MAJOR MOLECULAR MARKERS IN CONSERVATION GENETICS (SCHLOTTERER 2004)

Non invasive genetics: challenges and solutions

Molecular techniques are increasingly applied to the study of wildlife populations due to major advances in the field of molecular biology. In particular, the development of the polymerase chain reaction (PCR) (Mullis et al 1992), a technique that allows researchers to amplify DNA fragments even from minute amounts of DNA, has become a widespread tool in conservation biology. The combination of PCR and hypervariable DNA markers such as microsatellites and SNPs has allowed researchers to address a broad array of ecological and conservation genetic questions (Frankham et al 2002). Due to these technical advances, noninvasive genetic techniques (NGS) became an attractive approach to conservation biologists because they provide DNA samples of free-ranging animals minimizing or avoiding disturbance and negative effects of capturing (Taberlet et al 1999).Conservation biologists in particular have shown a deep interest in these techniques, which are now routinely used in forensic genetics and for investigating the biology and the genetic diversity of elusive, rare and/or endangered species avoiding any risks to impact their survival, their recapture rates or their population dynamics (Kohn & Wayne, 1997, Piggott & Taylor, 2003). NGS allows populations to be studied and censused (Frantz et al 2003, Broquet et al 2007) analysing DNA extracted from biological traces such as hairs (Woods et al 1999, Sloane et al 2000), faeces (Taberlet et al 1996, Gagneux et al 1997) and less direct sources of cells (urine and blood traces on snow (Valiere & Taberlet, 2000), sloughed skins (Bricker et al 1996), chewed food material containing buccal cells (Takenaka et al 1993), and bird feathers (Segelbacher, 2002) or egg shells (Pearce et al 1997).
The DNA extracted from samples collected noninvasively can provide species, individual, and sex identification (Taberlet et al 1999). NGS has increasingly recognized in the wildlife community, and it has been applied to estimate population size, trend, and density, assess genetic diversity and structure, detect hybridization and gene flow, and evaluate colonization histories, relatedness and mating systems for a variety of wildlife species, (Flagstad et al 2004, Piggott et al 2006). NGS is also expanding our ability to implement long-term monitoring programs for wildlife populations, including elusive species and small endangered populations, by allowing researchers to investigate demographic as well as genetic parameters.

However, NGS methods might present numerous potential problems which generally tend to limit the efficiency of this approach (Taberlet et al 1996, Broquet et al 2007). Non-invasively collected samples usually provide DNA extracts characterized by low target DNA concentration, low target DNA quality (Taberlet et al 1999), contaminations by alien DNA and various molecules that can disturb or inhibit the polymerase chain reaction (PCR) (Roon et al 2003; Broquet et al 2007), making it unreliable (Taberlet et al 1996, Gagneux et al 1997). High risk of contamination during DNA extraction and amplification and difficulties in amplifying degraded DNA are a direct consequence.
As amplification success and genotyping errors can be sensible to template DNA concentration and composition (Morin et al 2001), microsatellite genotypes from non-invasive samples can be affected by errors (Gagneux et al 1997, Smith et al 2000) such as allelic dropout (ADO) which is the stochastic failure of one allele to amplify for heterozygous individuals, producing false homozygotes (Navidi et al 1992, Constable et al 2001) and false alleles (‘misprinting’) which are artefacts of amplification products generated during the first steps of PCR that can be misinterpreted as true alleles (Goossens et al 1998).
Genotyping errors affect both the allele frequency estimates and the accurate discrimination of different genotypes. False estimates of allele frequency can create an artificial excess of homozygotes (Taberlet et al 1996, Gagneux et al 1997a), a false departure from Hardy–Weinberg equilibrium (Xu et al 2002), an overestimation of inbreeding rate (Gomes et al 1999, Taberlet et al 1999) or unreliable inferences about population substructures (Miller et al 2002). Erroneous genotypes can distort or overestimate population size estimates (Creel et al 2003), individual identification (Taberlet & Luikart, 1999; Paetkau, 2003) and parentage analysis (Miller et al 2002).
Many authors have recognized the complexities of non-invasive genotyping, and have developed methods to address these problems (Taberlet et al 1996, 1999; Morin et al 2001; Miller et al 2002): contaminations among samples could be avoided using dedicated rooms for extraction and amplification of low-DNA-content samples, while amplification from alien DNA could be avoided by using specific primers (Bradley & Vigilant, 2002). Numerous quality control protocols have been developed, including the adoption of *multiple-tube approaches* where the same DNA samples are amplified independently several times per locus (replicates) (Navidi et al 1992; Lucchini et al 2002;), comparison of genotypes obtained with those from matched blood or tissue (Parsons, 2001; Fernando et al 2003), strategic re-amplification at loci that present mismatches among replicates (Miller et al 2002) or among couples of similar genotypes (Woods et al 1999; Paetkau, 2003), pre-screening of samples for DNA quantity (Morin et al . 2001; Segelbacher, 2002) and the use of pilot studies and simulations (Taberlet et al 1996; Valiere et al. 2002). Anyway, all

these methods can involve a large extra experimental effort (Ewen et al 2000), increasing the consumables, costs and time required (Morin et al 2001).
Therefore, it is cheaper to conduct statistical tests on already available data. Commonly, the Hardy-Weinberg equilibrium test (Gomes et al 1999) is checked to reveal the homozygous excess resulting from either null alleles or allelic dropout.

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CHAPTER II - Thesis structure

This thesis deals with the application of molecular tools in brown bear conservation and management in Italy. Non-invasively collected brown bear samples, mostly hair and feces, were collected in the framework of pluriannual collaborations with management authorities responsible for the conservation of this species in Italy. Non-invasive samples were used in order to minimize disturbance in the natural habitat and facilitate the monitoring of this elusive and endangered species.
All samples were genetically analyzed in order to reconstruct individual genetic profiles and determining the sex of bears in the Alpine and Apennine brown bear populations. Standardized precautions were taken to deal with this challenging source of DNA.
Genetically identifying the largest possible number of individuals was the first step be achieved in order to monitor demographic and genetic trend of these two small populations. Unfortunately, while the whole Alpine area was covered by the sampling program, only bears from the peripherical area of the Apennines were collected, thus making impossible to study bears in central Italy at a population level.

As part of this PhD program, I have personally analyzed all bear samples collected between 2014 and 2015 in the Alps and Apennines. The genotypes identified, together with those obtained with previous (2001- 2013) and further analyses (2016), were used to built a genotype-databank, the starting point to achieve the objectives of this research project, which aims at a) providing an updated description of the demographic, spatial and genetic status on the reintroduced brown bear population in the Italian Alps and assist managers with conservation and management decisions and b) testing new genetic markers (microsatellites and SNPs) to increase the informativity content for individual identification, sex determination and parentage analysis on the Alpine and Apennine populations.

This PhD thesis has been divided into two sections: the first part is devoted to the application of molecular genetics to describe demographic trends, geographic distribution patterns and genetic status (**Chapter III**) of the Alpine population 15 years after the reintroduction program.
In order to achieve the first objective I: a) increased the number of STR markers (from 10 to 15 loci) in the Alpine population to raise the informativity content for population genetics studies, and possibly resolve uncertain parentage assignments; b) presented an annual overview of the demographic status of the Alpine population for a long-term monitoring program; c) measured the genetic diversity over generations, highlighting possible trends c) identified parentage relationships and provided a pedigree reconstruction, showing if there is an increase of inbreeding events over generations; d) estimated the effective population size; d) verified whether or not a connection between the reintroduced population in the central Alps and the Dinaric population was established.
I furthermore provided considerations for conservation and management of this species in the Alps, taking into account the emerged demographic, spatial and genetic aspects.
The second part is methodological and is about developing a new set of SNP markers to enhance the resolution power for population genetics analysis of the Alpine and Apennine populations (**Chapter IV**). In order to identify reliable and informative SNPs I a) tested the effectiveness of an existing SNP panel, developed for the Scandinavian brown bear populations, on the two Italian brown bear subspecies, identifying a set of SNPs that has potential for a SNP-based individual and sex identification system. I took into consideration the ascertainment bias that arises when transferring SNP markers across populations; and b) tested the selected subset of SNPs for parentage assignments in the Alpine population, comparing its resolution power with that derived from STRs (**Chapter V**).

CHAPTER III - History of a reintroduction: genetics as a tool for long-term monitoring of the brown bear population in the Italian Alps

ABSTRACT

In the 17th century, the brown bear was widely distributed from western Italian Alps to the Dinaric region in the Balkans. Direct human persecution and habitat fragmentation led to numerical contraction and geographical disruption into an Alpine and a Dinaric population. At the end of 1990's the Alpine brown bear population was considered biologically extinct, with only a few relict individuals. In 2000's a reintroduction program started with the aim of re-establishing a viable population in central Alps and a metapopulation in the Alpine region. Between 1999 and 2002, 10 bears captured in Slovenia were released in central Italian Alps. Between 2000 and 2016, non-invasive genetic monitoring based on individual genetic profiles was chosen for the post-release monitoring and a similar approach was used to monitor bear presence in eastern Italy, were males from the Dinaric population were dispersing. After 15 years from the reintroduction, we provide a general overview of the demographic, spatial and genetic status of the Alpine population and we evaluate whether or not connectivity between the Alpine and Dinaric populations has been reestablished. We analysed the genetic profile of 8577 bear samples (mainly non-invasively collected), 4757 of which (55.46%) successfully assigned to either released or newly identified genotypes. We identified 102 bears of the Alpine population and 23 Dinaric bears in eastern Italy. Females started to reproduce in 2002 and gave birth to 93 cubs (48 litters). The demographic trend was positive and the population expanded its distribution through the Alps. We found that the Alpine population is still genetically isolated, despite long distance male-biased dispersal has recently resulted in the partial geographical overlap of the Alpine and Dinaric populations. Moreover, reproductive success was strongly skewed towards a few male founders. As a consequence, we found the effective population size to be extremely small (minimum Ne= 6.4, maximum Ne= 8.0, depending on method used), allelic richness (Na) and expected heterozygosity (He) decreased (Na from 5.133 to 4.067; He from 0.702 to 0.618) in less than four generations, average coefficient of pairwise relatedness (Rxy) increased (from -0.129 in the first generation to -0.077 in the last generation). Results of this study are expected to have direct implications in management of the bear population in the Alps and can assist conservation efforts providing methodological and practical guidelines for large-scale monitoring programs following reintroductions using genetic sampling.

INTRODUCTION

In response to ever-increasing anthropogenic impacts on natural ecosystems, conservation scientists and agencies have implemented guidelines for monitoring environmental changes and the dynamics of endangered species and populations (Schwartz et al 2007). In this framework, many programs aimed at monitoring the consequences of anthropogenic pressures on wild populations took advantage of the potential of molecular genetic markers (Gibbs et al 1999, Nichols & Williams 2006). These markers can provide reliable information relevant to both demographic, ecological and evolutionary population dynamics of, more efficiently and cheaper than traditional monitoring approaches (Wayne and Morin 2004, Allendorf & Luikart 2006). Indeed, molecular markers can be used as a diagnostic tool for individual identification, the first step needed to further elaborate a number of demographic and genetic parameters, such as abundance, vital rates, genetic diversity and, when combined with spatial information, spatial movements and ranges (De Young & Honeycutt 2005, Koelewijn et al 2010). Genetic monitoring can also contribute in answering ecological issues on mating system, social behavior and dispersal patterns (Sugg et al 1996, Sigg et al 2005, Webster & Reichart 2005, Moore et al 2014) of elusive species.

In the last two decades, the ability of monitoring wild populations has expanded thanks to the use of non-invasive genetic sampling (NGS), i.e. extracting DNA from biological samples such as hairs, faeces, urine or other similar sources without handling, capturing or even observing the animals (Kohn & Wayne 1997, Taberlet et al 1999). Today, despite difficulties related to the low quality and quantity of DNA and the consequent risks of genotyping errors (Taberlet et al 1996, Gagneux et al 1997, Creel et al 2003), non-invasive samples are considered reliable sources of DNA (Piggot & Taylor 2003, Waits & Peatkau 2005, Von Thaden et al 2017). NGS is particularly appealing for long-term monitoring plans of endangered and elusive species like bears (Kendall et al 2009) because disturbance and negative effects of capturing are avoided (Cattet et al 2008). Thus, many recent publications concerning ecology, demography, population genetics or phylogeography of bears have used NGS methods (Gervasi eta l 2008, Pèrez et al 2009, Sawaya et al 2012, Schregel et al 2012, Tsaparis et al 2014).

The brown bear has suffered intense population bottlenecks in the past, mainly because of human persecution and habitat degradation, resulting in a contraction of both historical range and abundance. In the recent years, causes of population declines have been rectified in many cases, habitats are recovering in many areas, and laws are in place to prevent illegal actions such as poaching (Clark et al 2002), allowing many species to recolonize historical areas of presence.

Although wide dispersal capacities of male brown bears are well documented (Blanchard & Knight 1995, Stratman et al 2001), females do not disperse far away their mother's home ranges (Schwartz & Franzmann 1992). Also, bears have low reproductive rates (Bunnell & Tait 1981). These characteristics concur in limiting the natural colonization ability of this species (Hanski 1991, Hastings 1991), thus reintroductions have been often necessary to expedite this otherwise slow recolonization and re-establish extirpated populations (Swenson et al 2000).

In the 17th century the brown bear was still considered widely distributed from western Italian Alps to the Dinaric region in the Balkans. The numerical decline started during the 18th century (Duprè et al 2000, Mustoni et al 2003) and in the 20th century bears were almost eradicated from entire southern Europe (Swenson et al 2000). The numerical decline led to a geographical contraction through the Alps. As a result, the Alpine population disrupted into two small isolated groups in central and eastern Italian Alps (Dupré et al 2000). The contraction continued and, by the 1990's, only a few relict individuals survived in central Italian Alps in Trentino region, thus this population was considered biologically extinct (Duprè et al 2000, Mustoni et al 2003The brown bear last surviving in the eastern Italian Alps was killed in 1911.

Several conservation actions have been taken in the last two decades to help the population bear recovery in Italy: in 2000's a reintroduction program started with the aim of re-establishing a viable population in central Alps (50-90 bears)(De Barba et al 2010) and a metapopulation in the Alpine region (Mustoni et al 2003). Between 1999 and 2002, 10 bears captured in Slovenia were released in the Trentino region and were initially radio-monitored. The program was co-funded by the European Union through the "LIFE Ursus" project. Challenges in continuous monitoring the reintroduced bears with traditional field methods became evident, thus a genetic monitoring program was implemented in 2002. A parallel genetic monitoring program was started in Eastern Italy in 2001, when the recovery of the numerical consistency of the Dinaric bear population in Croatia and Slovenia led to its progressive expansion to the north-west, with consequent recolonization of Italian and Austrian Alps and Prealps. The natural expansion of bears from Dinaric mountains into eastern Italy is now promoted through the "LIFE Dinalps" program: a multidisciplinary approach is used to look into this issue and try to understand the social and physical barriers to expansion and the habitat corridors that need to be protected.

In-depth monitoring of bears is critical to assess reintroduction outcomes and to ensure prompt actions for improving status and probability of population persistence (Miller et al 1999). Monitoring is mandatory under the European Union Directive (1992), and recommended by countries' s action plans and guidelines (AA.VV.a 2007, Servheen et al 1999, Swenson et al 2000; AA.VV.b 2007, IUCN 1995).

In this study, we obtained demographic information (population abundance, reproductive, recruitment and mortality rates, sex ratio and age structure) from molecular analyses of biological samples collected between 2000 and 2016 from the brown bear populations in the Italian Alps. Moreover, we reconstructed the spatial distribution and individual movements of brown bears to delineate patterns of geographic expansion from the reintroduction area in central Italian Alps and from eastern Italy borders, where Dinaric brown bears of population are dispersing. We aimed to evaluate dispersal behavior and to assess whether or not connectivity between the two isolated populations have been reestablished. Mating behavior, that can be strongly influenced by the small number of available mates, is investigated, especially because selective mating can have strong effects on genetic structure, effective population size, and inbreeding rates (Chesser 1991, Sigg et al 2005, Sugg et al 1996, Vonholdt et al 2008).

Population genetic parameters, such as genetic variability, inbreeding and the related effective population size, are also examined. In small and isolated populations, especially those descending from a limited number of founders (Tokarska et al 2009), inbreeding events are more likely to occur, resulting in most individuals within the population to be related after a few generations (Hedrick 2000). Inbreeding events rapidly lead to reduced genetic diversity through loss and fixation of alleles, increase in homozygosity, and shifts in allele frequencies (Ralls et al 1988, Allendorf, Luikart and Aitken 2013). Inbred individuals may have reduced fitness and higher mortality rates in comparison with non-inbred individuals. Homozygosity leads to the expression of deleterious recessive alleles and reduction of genetic diversity decreases long-term adaptative potential (Allendorf, Luikart and Aitken, 2013). Lower fitness and higher mortality contribute to a further numerical decline of the population, which can enter into an "extinction vortex" (Blomqvist et al 2010).

Finally, we wanted to assess the power the sampling protocol after 15 years from the reintroduction. Results will provide important information for planning future conservation actions for bear population in the Italian Alps as well as a model for other reintroduction programs.

MATERIAL AND METHODS

Study area

The study area covers almost the entire north-eastern territory of the Italian Alps. It includes 4 administrative districts, from west to east: Lombardia (LOMB), Trentino-Alto Adige (TN-AD), Veneto (VEN) and Friuli Venezia Giulia (FVG) (Fig. 1 – blue line). Moreover, a few samples were collected outside of the Italian border, in neighboring countries (Austria, Switzerland, and Germany). It is a predominantly mountain region, with the elevation ranging from 100 m to 3500 m, comprising a variety of vegetation belts such as submediterranean, submontane, montane, subalpine and alpine (Dupré et al 2000, Mustoni et al 2003). Deciduous forests, dominant in the area below 1000 m, are replaced by conifers at higher elevations (i.e., 1000 – 2000 m). Bushes and herbaceous plants take place in woodlands above 2000 m. Several valleys, carved by rivers, torrents, lakes, cross the study area from the northern alpine region to the southern subalpine and submediterranean area. Two major highways and railways run N-S through the study area along the urbanized Adige and Tagliamento river valleys (fig 3.1). Human density is high in the alpine region (e.g. 81 inhabitants/km^2 in the Trento province) and mostly concentrated in the valleys, where the economy is dominated by tourism and agriculture. Diffuse farming and livestock are typical at medium altitude areas (500-1000 m), while the density of residents is lower and concentrated along valleys at higher elevations.

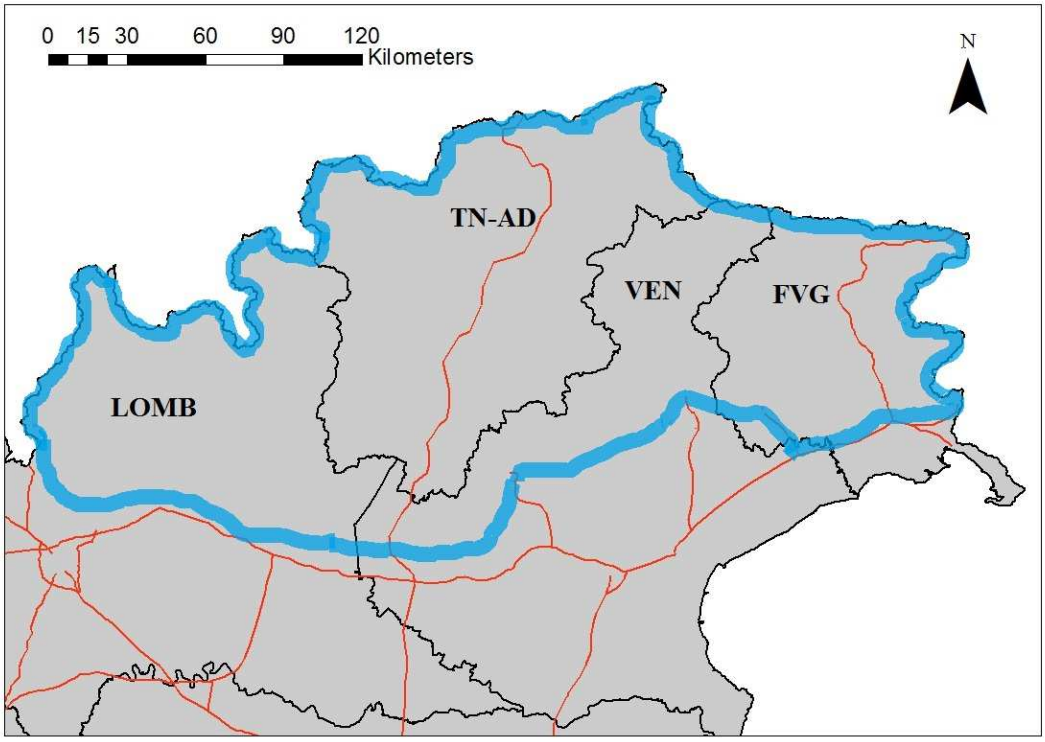


FIG 3. 1 - STUDY AREA (IN BLUE). THE RED LINE REPRESENTS MAJOR ROADS, WHILE BLACK LINES REPRESENT THE FOUR ADMINISTRATIVE REGIONS (LOMB=LOMBARDY; TN-AD=TRENTINO ALTO ADIGE; VEN=VENETO; FVG=FRIULI-VENEZIA GIULIA).

Sampling procedures

Different sampling procedures were used, depending on the administrative districts, but standardized and calibrated according to the local context and following guidelines provided by the interregional action plan for the conservation of brown bears in the Italian Alps (AA.VV 2010 - PACOBACE): ascertaining bear dispersal through opportunistic genetic sampling was the only objective in non-colonized areas, whereas an integration of systematic and opportunistic approach with different sampling methodologies was used to detect the largest possible number of individuals (including cubs) in areas of stable presence. Both invasive (tissues, bones, teeth, blood, and hairs) and non-invasive samples (hairs, feces, saliva, urine) were collected. Baited and unbaited hair-traps were used following Woods et al 1999, and transect sampling was conducted to search for fecal samples. Other samples were collected during inspections at damaged livestocks and beehives, when bears were found dead (e.g. killed by vehicle collisions

or poached) or during captures (e.g. for GPS-collar positioning). (see De Barba et al 2010 for a review of sampling methods). All samples were collected using sterilized forceps or latex gloves and placed in coin envelops stored in silica dissecant or alternatively in 96% ethanol, depending on sample type. Any remaining of the sample was removed to avoid repeated sampling. Geographic coordinates were recorded when possible.
Field monitoring methods, such as GPS-VHF monitoring, photo-trapping and direct counts of females with cubs, were also carried out together with genetic sampling in order to use an integrated approach. This kind of data is not reported in this study, but are used to confirm genetic results.

Genetic methods

All samples collected between 2000 and 2016 were genetically analyzed. From 2010 to 2016, genetic analyses were performed at the Italian Institute for Environmental Protection and Research (ISPRA, Italy) and before 2010, at the University of Idaho upon obtaining CITES permits (De Barba et al 2010). Genetic methods used until 2009 are described in detail in De Barba et al. (2010). From 2010 onwards, given the growing number of samples collected, methods were slightly modified to speed up the analysis. The following describes the major protocol changes: DNA extractions started to be performed using a robotic platform on 48 or 96 well plates (Tecan Freedom EVO® 100 Liquid Handling Platform) and new commercial kits, the Quick-g DNATM miniprep D3025, the ZR-96 Quick-g DNATM (Zymo Research, Freiburg, Germany) and the DNeasy® 96 Blood & Tissue Kit (QIAGEN, Hilden, Germany). To minimize risks of contamination, DNA extractions were performed in dedicated rooms, free of sources of exogenous contaminant DNA, and a negative controls were added at each extraction. All DNA samples were eluted in 200 µL of elution buffer and stored at -20°.
A total of ten microsatellite loci were used for individual identification: the number of multiplex amplification was reduced from 3 to 2 (called M1 and M2). Multiplex M1 was used as a screening of sample quality (5 loci) to exclude samples from non-target species or of poor quality DNA (Peatkau 2003): a multi-tube approach (4 replicates per locus) for non-invasively collected samples was used (Taberlet et al. 1996, Adams and Waits 2007). Samples were kept for further analysis if had scorable results at ≥ 50% of loci. Selected samples were amplified four times at the remaining 5 loci (M2) to complete the individual multilocus genotype. Sex was established through the analysis of the amelogenin gene (Ennis and Gallagher, 1994).
During the first 15 years, 10 loci were sufficient for parentage assessment. Subsequently, because of loss of released individuals, the occurrence of offspring and the increasing relatedness among individuals, the number of microsatellite loci had to be increased. Starting from 2014, a third multiplex comprising 5 more loci (M3) was added to enhance the marker informativity content for parentage analysis, while the SRY locus (Fechner 1996) was used to confirm sex determination. All individuals identified from 2000 to 2016 were processed for multiplex M3. A list of all 15 autosomal loci with primer sequences, multiplex amplifications, allelic size range, and references is provided in table 3.1.
The Qiagen Multiplex PCR Kit was used for amplification in the three PCR reactions: The 8 µl PCR reaction consists of 0.80 µl 10X reaction buffer, 0.80 µl BSA (0.2%), 0.80 µl DNTPs (2.5 mM), 0.04 µl Taq polymerase (5U/ µl), 2 µl DNA,0.14 µl G10M, 0.12 µl G10P, 0.12 µl Mu11, 0.06 µl cxx20, 0.10 µl Mu15 in M1, 0.15 µl G10X, 0.09 µl Mu59, 0.10 µl G1D, 0.09 µl Mu23, 0.07 µl Mu50, 0.05 µl AMG in M2, 0.12 µl G10C, 0.10 µl Mu09, 0.10 µl G10H, 0.12 µl G10L, 0.18 µl Mu10 and 0.08 µl SRY in M3. The PCR profile had an initial denaturation step of 2 minutes at 94 C°, followed by 55 cycles of 15 seconds 94 C°, 30 seconds at 52,5 C°, a final extension of 30 seconds at 72 C°, and 10 minutes at 72 C° followed by ten minutes at 4 C°. Negative and positive controls were added to each group of reactions to test for contamination and calibrate allele calling among different runs. PCR products from each multiplex were added to 10 µl mix of formamide and 20 µl LIZ500 size standard and run in 16 separate capillaries on the ABI Prism 3130xl Genetic Analyzer (Applied Biosystem). PCR products were scored using GeneMapper software (version 4.0, Applied Biosystems - Life Technologies, Carlsbad, California, USA).

Genotyping reliability and marker suitability

We estimated the power of the former 10 sex-independent microsatellite markers in distinguishing individuals through the probability of identity test (PID), according to Waits et al. 2001. We further compared values obtained by adding 5 loci. To overcome the bias caused by the presence of closely related individuals in the population, we also calculated the equivalent probability for pairs of siblings (PIDsibs), which are more likely to share identical genotype by chance (Waits et al. 2001). Allele frequencies, PID and PIDsibs values and number of mismatches among different genotypes (MM) were calculated using GeneAlEx 6.502, separately for bears belonging to the reintroduced population in the central Italian Alps (founders and their offspring) and those dispersing from the Dinaric population and sampled in eastern Italy. The probability of exclusion, defined as the probability of excluding a random individual from the population given alleles of the offspring and the mother, with both parents known (P1) and only one parent known (P2) was calculated only for bears in central Italian Alps. The number of mismatches was used to scrutinize the similarity of unique genotypes by looking at the presence of genotypes with only 1-2 mismatches (Peatkau 2003).
Presence of genotypes errors (allelic drop-outs and false alleles) was regularly checked using GIMLET (Valière 2002), and four more PCR replicates at single loci were added in case of genotyping errors or missing data. However, given the enormous number of replicates in the total dataset, we re-calculated genotyping errors for a subset of randomly selected non-invasive and invasive successfully genotyped samples in order to provide average values. Multilocus consensus genotypes with a reliability R score ≤95% calculated with RELIOTYPE (Miller et al. 2002) were discarded, while others were kept for further analysis.

Locus	PCR primers (5'-3')	M	Dye	Size range	Reference
Mu11-F	AAGTAATTGGTGAAATGACAG	M1	Hex	75-92	Taberlet et al 1997
Mu11-R	GAACCCTTCACCGAAAATC				Taberlet et al 1997
G10M-FIm	GTTTGCCTCTTTGKCTACTGGA	M1	Fam	106-125	Taberlet et al 1997
G10M-Rm	CAAATAATTTAAATGCATCCCAGGGG				Taberlet et al 1997
cxx20-F	AGCAACCCCTCCCATTTACT	M1	Ned	112-135	Ostrander et al 2001
cxx20-R	TTGTCTGAATAGTCCTCTGCG				Ostrander et al 2001
Mu15-F	GCCTGACCATCCAACATC	M1	Hex	125-135	Taberlet et al 1997
Mu15-R	AAATAAGGGAGGCTTGGGT				Taberlet et al 1997
G10P-Fp	AGTTTTACATAGGAGGAAGAA	M1	Fam	149-179	Paetkau & Strobeck 1995
G10P-Rp	TCATGTGGGGAAATACTCTGAA				Paetkau et al 1998
Mu50Fb	GTCTCTGTCATTTCCCCATC	M2	Hex	77-102	Bellemain & Taberlet 2004
Mu50Rib	AACCTGGAACAAAAATTAACAC				Bellemain & Taberlet 2004
G1D-FIm	CCATCTCTCTTTTCCTTTAGGG	M2	Pet	96-118	Taberlet et al 1997
G1D-R	CTACTCTTCCTACTCTTTAAGAG				Taberlet et al 1997
Mu23-F	GCCTGTGTGCTATTTTATCC	M2	Fam	118-128	Taberlet et al 1997
Mu23-RI	AATGGGTTTCTTGTTTAATTAC				Taberlet et 1997
Mu59-F	GCTCCTTTGGGACATTGTAA	M2	Ned	106-119	Taberlet et al 1997
Mu59-Rib	TGACTGTCACCAGCAGGAG				Bellemain & Taberlet 2004
G10X-F	CCCTGGTAACCACAAATCTCT	M2	Fam	126-156	Taberlet et al 1997
G10X-R	TCAGTTATCTGTGAAATCAAAA				Paetkau et al 1998
Amel4	AGAGGCAGGTCAGGAAGCAT	M2	Fam	155-215	Ennis and Gallagher 1994
SE47	CAGCCAAACCTCCCTCTGC				Ennis and Gallagher 1994
G10C_F	AAAGCAGAAGGCCTTGATTTCCTG	M3	Hex	97-116	Taberlet et al 1997
G10C_R	GGGACATAAACACCGAGACAGC				Taberlet et al 1997
G10H_F	CAACAAGAAGACCACTGTAA	M3	Fam	221-257	Paetkau & Strobeck 1995
G10H_R	AGAGACCACCAAGTAGGATA				Paetkau & Strobeck 1995
G10L_Fi	ACTGATTTTATTCACATTTCCC	M3	Pet	156-166	Paetkau & Strobeck 1995
G10L_R	GATACAGAAACCTACCCATGCG				Paetkau & Strobeck 1995
Mu09_F	AGCCACTTTGTAAGGAGTAGT	M3	Hex	174-206	Taberlet et al 1997
Mu09_R	ATATAGCAGCATATTTTTGGCT				Taberlet et al 1997
Mu10_F	ATTCAGATTTCATCAGTTTGACA	M3	Ned	114-130	Bellemain & Taberlet 2004
Mu10_R	TCAGCATAGTTACACAAATCTCC				Bellemain & Taberlet 2004
SRY_F	GAACGCATTCTTGGTGTGGTC	M3	Fem	75-75	Fechner 1996
SRY_R	TGATCTCTGAGTTTTGCATTTG				Fechner 1996

TABLE 3. 1 - LIST OF ALL 15 AUTOSOMAL LOCI AND PRIMER SEQUENCES, LIST OF MULTIPLEX AMPLIFICATIONS, ALLELIC SIZE RANGE AND REFERENCES

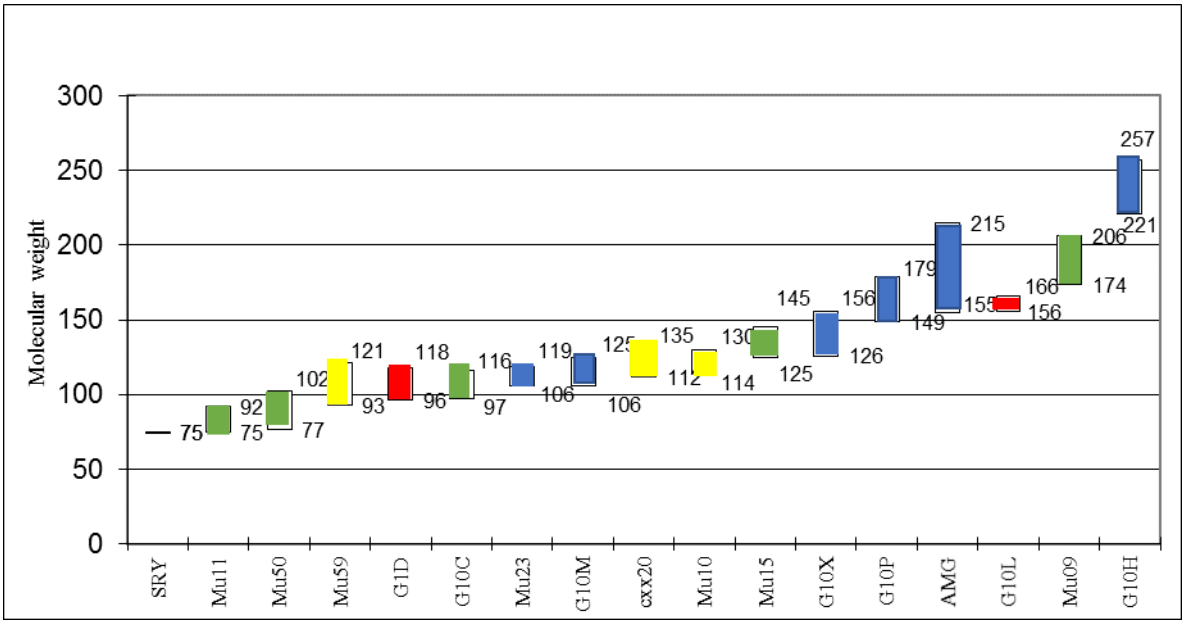


FIG 3. 2 – SCHEMATIC REPRESENTATION OF MOLECULAR WEIGHTS OF AMPLIFIED LOCI AND DYE COLORS

Genetic databank

Since the Alpine population in central Alps was small at the beginning of the project (9 founders) and isolated, and immigration from the Dinaric population was unlikely, it was effectively a close population. At the start of the reintroduction project a reference genetic databank of founders (individual multilocus sexed genotypes) was constructed using blood and tissue samples (De Barba et al 2010), consisting of. So that, a reference genetic database was developed and updated in the subsequent years adding newly identified bears, the offspring of the founders. The genetic databank was developed using a "matching approach" to assign the founder samples to the reference genotypes (Adam and Waits 2007), using GeneAlEx 6.502 (Peakall & Smouse 2006). New individuals (new genotypes; the offspring) were identified if a) a multilocus genetic profile did not match any existing profile from previous years, and b) a genetic profile was probabilistically assigned to a couple of putative parents. An independent but similar genetic databank was developed for the Dinaric bears dispersing in the eastern Italian Alps. Since males have great dispersal capacity, we tested the hypothesis that bears of the Dinaric population, sampled in eastern Italy, could have dispersed to central Italian Alps. We used the matching approach analyzing bears sampled in central Alps and eastern Italy together to determine if there were matching genotypes.

Genotyping success among years, sampling approach (invasive/non-invasive), and type of organic material (tissue, hairs, tooth, bone, blood, feces, saliva, urine) was calculated as the number of successfully genotyped samples over the number of all samples collected and analyzed.

Parentage analysis and pedigree reconstruction

Parentage relationships was evaluated using FRANz software v.2 (Riester et al. 2009), a common likelihood-based package which uses Markov chain Monte Carlo sampling to assign parentage. FRANz calculates LOD scores for each possible parent-offspring pairing and uses this value to assign parentage across a group of offspring. A negative LOD score means that the candidate parent is less likely to be the true parent than not the true parent. A negative LOD score indicates that a candidate parent mismatches at one or more loci. Negative LOD scores can also occur when the candidate parent and offspring share very common alleles at every locus. Conversely, a positive LOD score means that the candidate parent is more likely to be the true parent than not the true parent. The actual true parent almost always has a positive LOD score. Posterior probabilities, which are the fractions of sampled pedigrees with that parentage assignment.

Parentage tests were performed every year for every newly genetically identified bear. Year of birth of all putative parents in the dataset was determined with previous parentage assignments and included in the analysis. We considered putative parents all bears genetically detected in the study area and belonging to the reintroduced population in central Italian Alps (founders and their offspring). Parentage analysis was routinely performed only for bears of the central Alpine population and not for bears sampled in the eastern Alpine area since genotypes of bears of

the original Dinaric populations are not at disposal and identification of parentage relationships of Eastern bears is outside the scope of this study. However, one test for parentage analysis was performed including bears dispersing from the Dinaric population as putative parents, in order to verify if gene flow was occurring between the two disjoint nuclei in the central and eastern Italian Alps.
FRANz is able to take the year of birth into consideration to calculate when putative parents enter the reproductive age, that we set to 3 years for females and 4 years for males, based on empirical data on this population (see results). Sex information, derived from AMG and SRY data, was included for putative parents and offspring. The empirically determined mean typing error of 0.002 among replicates and allele frequencies were specified. A second software for parentage analysis (Colony v. 2.0.6.4, Jones and Wang 2010) was used to confirm or reject parentage assignments. Uncertain parental relationships were determined when discordances in parentage assignments between the two softwares were detected. A complete pedigree of individuals was drawn using Graphviz v. 2.34.

Monitoring history, demographic and spatial distribution

The complete monitoring history of the population was used to construct a detailed life table for each sampled bear, graphically described using the following legenda:

.	Not born
1	Alive and present in the population
*	Year of birth of a cub genetically determined year/s after
2	Presence estimated
⍉?	Hypothized year of death (not sampled since 2012)
⍉	Year of death
†	Dead
Θ	Captivated
↗	Emigrated
?	Disappeared
	Year of birth
	Year of reproduction
	Juvenile (M:1-4 years; F:1-3 years)
	Adult (M:more than 4 years; F: more than 3 years)

TABLE 3.3 – LIFE TABLE LEGENDA

Demographic data were directly derived or estimated from the complete monitoring history of each bear and identification of parent-offspring relationships. For example, an adult bear detected in 2013 and 2015 but not in 2014, was considered present in 2014 (symbol “2”). This is likely true only for bears from central Italian Alps, and not for those sampled in the eastern Italian border, where bears often move towards Slovenia to their territories of origin. Given the lack of homogeneity in information availability, bears belonging to the two disjoint nuclei in the Italian Alps were treated separately.
Year of birth and year of reproduction were established on the basis of parentage assignments, but field and observational data were used to refine it: for example, year of birth was adjusted when a bear genetically identified for the first time in 2016, was known to be 1 year old or older from field data (symbol ”*”).
Cubs were defined of age 0, and the transition to adulthoods (3 years for females and 4years for males) was determined by the onset of sexual maturity for both sexes, while juveniles are bears of age between 1 and 3 or 4 years, depending on sex.
The ascertained year of death (symbol “⍉”), was reported only when the bear carcass was found or the bear was legally killed, while hypothized year of death (symbol “⍉?”) was determined when the bear was not detected with genetic sampling for ≥ 2 consecutive years. This criterion was chosen because data show that bears not detected for two subsequent years were never detected afterward.Years following death are indicated with a cross (symbol “†”).
Bears were considered emigrated from the core area in Trentino (symbol “↗”) when they had crossed the Tagliamento river and never came back, while they were considered “disappeared” when not detected in the last 1 or 2 years. Further information will be needed to determine the fate of disappeared bears, since they could be dead, emigrated, or simply not sampled in the last years. A few bears were captivated (symbol “Θ”) for public safety (e.g. problematic bears particularly prone to human aggression).
Data derived from individual identification and parentage analysis included: yearly measures of the population size (minimum counts), growth rate, age structure, sex ratio for adults and cubs at birth, number of reproductive events

(number of litters), number of male and females reproducing, number of cubs, survival rates among cubs, juveniles and adults, and number of mortality events.
The spatial pattern of the population and individual movements across the study area were evaluated entering geographic coordinates of bear samples in a GIS database and visualized using ArcView v.10.2 (ESRI) software.

Census population size

The estimate of population size from non-invasive samples (N_o) was obtained using the R package "capwire" v1.1.4 (Pennell and Miller, 2013), which has been developed to handle capture-recapture data collected in small closed populations, in the presence of marked capture heterogeneity. To avoid strong violation of the assumption of population closure, we analyzed separately the genotypes identified each year, from 2003 to 2013, using the software default parameter values. Capwire implements several demographic models and includes functions to perform a likelihood ratio test to choose between models, perform parametric bootstrapping to obtain confidence intervals and multiple functions to simulate data. We tested two models: the ECM (Equal Capture Model), an equal probability model in which each individual has the same probability $1/N_o$ to be captured, and the TIRMPartition (Two Innate Rates Model After Partitioning Data), a model that divides count data into three groups based on PART algorithm and in which the population is a mixture of hard and easy sampling individuals. The lower two groups are retained for the population estimation procedure implemented in capwire, while the upper group is excluded from the estimation procedure.

Genetic diversity, inbreeding and effective population size

GeneAlEx 6.502 was used to calculate several parameters describing nuclear genetic diversity in the reintroduced population: allele frequencies, mean number of alleles per locus (Na), effective number of alleles (Nea), Shannon's information index (I), observed (Ho) and expected unbiased (uHe) heterozygosity and fixation index (F). All these measures were calculated per year and generation, in order to highlight possible trends in genetic variability.
Arlequin v. 3.5 (Excoffier et al 2010) was used to assess deviations from Hardy–Weinberg equilibrium (HWE) and to calculate pairwise linkage disequilibrium (LD) by generations, both with an adjusted p-value corresponding to 0.05 after the Holm–Bonferroni correction for multiple tests (Holm, 1979). Since we are relatively certain that the brown bear population in the Italian Alps is reproductively isolated, we expect that the linkage disequilibrium (LD) at neutral loci may grow as a result of genetic drift (Nei and Tajima, 1981).
We also used GeneAlEx 6.502 to perform principal component analysis (PCA). In conventional PCA, markers are treated as variables and sampled individuals are plotted into a bi- or tri-dimesional Cartesian's coordinate system, spanned by the top principal components (PCs), which summarize data information content. Because top PCs reflects variations due to population structure in the dataset, individuals with similar multilocus genotypes will form a cluster in this subspace. The pattern of produced scatter plots (PC-plots) reflects inter-population relationships or within-population structures (Ma & Amos, 2012). We analyzed all Dinaric bears sampled in eastern Italy together with reintroduced bears and their offspring to evaluate if there was any kind of population sub-structure between the original and the reintroduced population. We created one PC-plot per generation in order to highlight possible changes over time. Genetic markers provide information about relatedness between individuals (Thompson 1975; Lynch 1988; Queller & Goodnight 1989; Blouin et al. 1996; Ritland 1996a; Goodnight & Queller 1999; Lynch & Ritland 1999).We calculated pairwise relatedness coefficients (Rxy), interpreted as non-random mating within sub-groups, per generation using GeneAlEx 6.502. The coefficient of relatedness (*r*xy) measures the expected proportion of shared alleles between pairs of individuals that are identical by descent (IBD) (Blouin 2003).
We used three different estimators: RI (Ritland 1996), LRM (Lynch & Ritland 1999) and QGM (Queller and Goodnight 1989). Linch & Ritland's estimator Ri values were multiplied by 2 to give a maximum value of 1 and minimum of -1 (default max is 0.5), this to standardize LR's range with the other two estimators.
Genetic drift in small populations can generate linkage disequilibrium (LD) due to stochastic fluctuations in allele frequency occurring from generation to generation (Hill 1981; Waples 1991). We used NeEstimator v.2.01 (Peel et al. 2004), which uses Burrow's composite measure of disequilibrium (Bartley et al. 1992) to estimate r^2 (the mean squared correlation of allele frequencies at different gene loci) and then contemporary (year 2016) effective population size (Ne). We also provided simulations for slopes of heterozygosity loss over years and generations, which we calculated for 100 and 500 years, using the R package "NEff" v1.0 (Grimm, Gruber & Henle, 2013).

RESULTS

Sample collection and microsatellite genotyping

A total of 8487 samples were collected during 2000-2008 using non-invasive approaches. In addition, 90 samples were obtained non invasively from dead individuals or captured bears. The majority of samples were collected, in the core area, where bears were reintroduced (Tn-Ad – 7305 samples), followed by the eastern Italian border (FVG – 804 samples), and other regions in between, (Ven - 236 samples, Lomb – 208 samples). A few samples (24) were collected outside the Italian border, in Austria, Germany, and Switzerland. Most of the samples collected non-invasively were hairs (6181), followed by feces (2292).

Microsatellite genotyping success rate ranged from 41,74% (2003) to 100% (2000 first year of the monitoring program). The 100% genotyping success in 2000 is likely due to the sampling of only 3 invasive samples. 66,35 % and 40,69% of invasive and non-invasive samples were successfully genotyped, respectively. Among non-invasive samples, genotyping success was relatively high for hairs and (65.38%) and saliva (54.55%), low for feces (28.23%) and null for urin. Among invasive samples, 100% of blood samples were successfully genotyped, followed by hairs (70.21%), tissue (61.54%), and bones and teeth (50%).

All single values per year, administrative district and sample type, are reported in tables 3.4 and 3.5. On average, a unique genotype was identified on 55.43% of samples.

Year	Lomb	Tn-Ad	Ven	Fvg	Aus	Ger	Swiss	N sampled	N successfully genotyped	% Genotyping success
2000		3						3	3	100.00%
2001		1		6				7	5	71.43%
2002		15		4				19	14	73.68%
2003		357						357	149	41.74%
2004		472		22				494	277	56.07%
2005	1	323		64	3		2	393	242	61.58%
2006		419		31		1		451	284	62.97%
2007	3	317	11	86			11	428	236	55.14%
2008	10	435	5	67				517	285	55.13%
2009	18	333	25	20	3			399	202	50.63%
2010	23	698	21	65				807	431	53.41%
2011	22	616	37	107				782	378	48.34%
2012	36	616	57	75	3			787	434	55.15%
2013	7	785	22	95				909	563	61.94%
2014	36	505	25	65				631	378	59.90%
2015	31	726	11	50				818	450	55.01%
2016	21	684	22	47	1			775	426	54.97%
TOT	**208**	**7305**	**236**	**804**	**10**	**1**	**13**	**8577**	**4757**	**55.46%**

TABLE 3.4 – NUMBER OF SAMPLES COLLECTED AND SUCCESSFULLY GENOTYPED PER YEAR OF SAMPLING AND ADMINISTRATIVE DISTRICT

	Invasive						Non invasive					N samples TOT
Year	**Tissue**	**Hairs**	**Teeth**	**Bone**	**Blood**	**TOT**	**Hairs**	**Feces**	**Saliv**	**Urin**	**TOT**	
2000		3				3					0	**3**
2001		7				7					0	**7**
2002		6			2	8	11				11	**19**
2003	1		3	1		5	289	63			352	**357**
2004						0	333	161			494	**494**
2005		5				5	293	95			388	**393**
2006	3	1				4	363	84			447	**451**
2007		4			1	5	279	144			423	**428**
2008	4	8			1	13	254	249		1	504	**517**
2009	1	1				2	165	232			397	**399**
2010	1					1	577	229			806	**807**
2011	1	1				2	518	258	3	1	780	**782**
2012	7	1	2			10	575	202			777	**787**
2013						0	744	165			909	**909**
2014	2	4			1	7	490	132	1	1	624	**631**
2015	1	4	3	3		11	639	163	5		807	**818**
2016	5	2				7	651	115	2		768	**775**
N sampled	**26**	**47**	**8**	**4**	**5**	**90**	**6181**	**2292**	**11**	**3**	**8487**	**8577**
N genotyped	**16**	**33**	**4**	**2**	**5**	**60**	**4041**	**647**	**6**	**0**	**4694**	**4754**
% succ	**61.54**	**70.21%**	**50.00**	**50.00**	**100.00**	**66.67**	**65.38**	**28.23**	**54.55**	**0.00**	**55.31**	**55.43%**
Mean	**66.35%**						**40.69%**					

TABLE 3. 5 - NUMBER OF SAMPLES COLLECTED AND SUCCESSFULLY GENOTYPED PER SAMPLE TYPOLOGY

Values of the probability of identity (PID) for increasing locus combination were $4.4x10^{-13}$ and $2.0x10^{-16}$ for the central Alpine population and for bears sampled in eastern Italy, respectively, using the all 15 microsatellite loci. The probability of identity among siblings (PIDsibs) ranged from $6.9x10^{-6}$ for the central Alpine population to$7.8x10^{-7}$ for the eastern population, providing high discriminatory power in all cases. The former set of 10 microsatellite marker showed higher values for both PID and PIDsibs: $4.0x10^{-10}$ and $1.4x10^{-4}$ respectively for the alpine population, while $5.7x10^{-12}$ and $5.6x10^{-5}$ for bears sampled in eastern Italy, belonging to the Dinaric population.

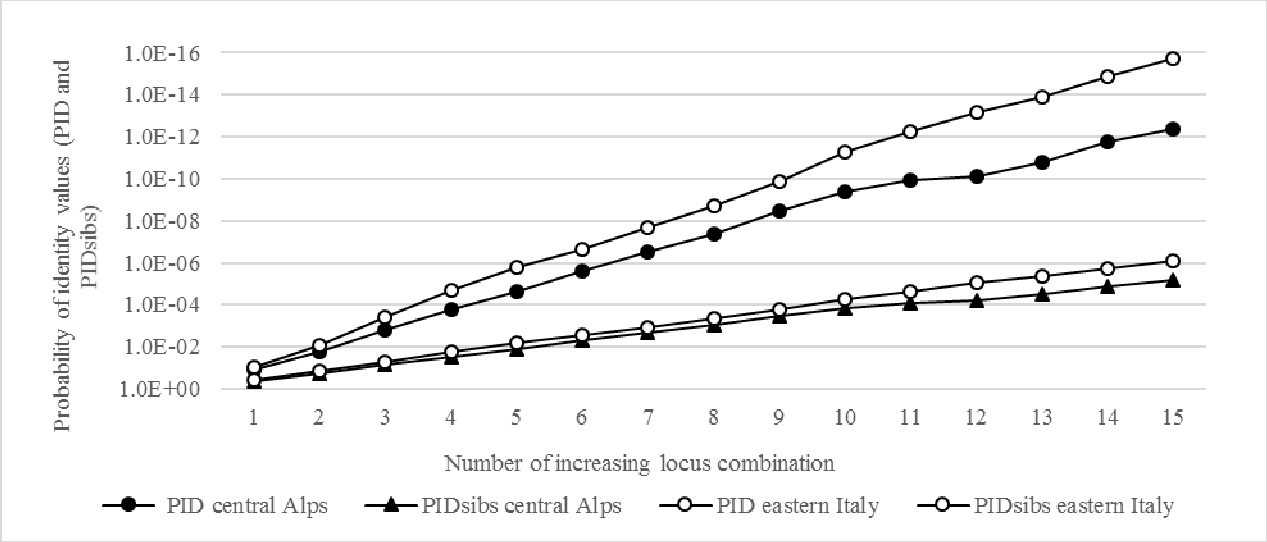


FIGURA 3.3 – PID AND PID VALUES FOR INCREASING LOCUS COMBINATION OF 15 MICROSATELLITE MARKERS

The exclusion power of the marker set was high since P1 (both parents known) was 0.999, and P2 (only one parent known) was 0.992. There were no 1-2 MM-pairs in the 2001-2016 15-loci reference genotypes either in the central Alpine population, with the exception of two bears from the central Alps (F4 and M20), which have all but two alleles in common. Moreover, there are a few reference genotypes in the central Alps with all but 3 alleles in common (F4 and M36; M36 and M40), while reference genotypes of bears sampled in eastern Italy are extremely different, with a maximum of 7 alleles in common.

Among a group of randomly selected samples, genotyping error rate due to allelic drop-out was 3.5% – 9.7% for invasive samples and 7.3% - 21.5% for non-invasive samples. Error rate due to false alleles was 0.13% – 4.5% for invasive samples and 0.16% - 9.3% for non invasive samples.

Among 4757 successfully genotyped samples, a total of 102 bears were genetically identified in central Italian Alps in the 2000-2016 period, 9 of which were founders of the reintroduced population (Table 3.6). One founder, named Masun, was not genotyped because dispersed outside of the translocation area soon after his release before the genetic monitoring program was started. All genotypes were processed for sex determination using AMG and SRY, no incongruent results were found among these two sex-dependent markers. In addition, more 23 bears dispersing from the Dinaric population were sampled in eastern Italy, in Friuli-Venezia Giulia, were an independent genetic monitoring program was carried out in the same period (Table 3.7). When searching for matching genotypes among samples from central Italian Alps and eastern Alps, we found that 6 male bears (indicated with the symbol "*") born in central Italian Alps dispersed to eastern Italy, where they were sampled starting from 2009. One case of immigration was detected: the bear named M5, which was sampled in central Italian Alps for the first time, was actually belonging to the Dinaric population. Its origin was determined on the basis of parentage analysis (no parents found in the dataset) and field data (it has been equipped with a GPS-collar in Slovenia).

The following life table is constructed on the basis of genetic sampling, but field data were used to refine it: years of death could not have been reported without carcasses, and many years of birth do not coincide with first year of genetic sampling because mothers were sampled and observed with cubs in the previous year, therefore cubs were assumed to be born in the year prior to their detection with genetic sampling.

Since bears sampled in eastern Italy were born in Slovenia, no data on year of birth, age or death (with the exception of Gen16) are available. Thus, the only year of presence is reported.

N	Bear ID	Sex	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
1	Masun	male	1	⌀?	†	†	†	†	†	†	†	†	†	†	†	†	†	†	†
2	Gasper	male	.	.	1	1	1	1	1	1	1	1	1	1	1	1	⌀	†	†
3	Brenta	female	.	.	1	1	1	1	⌀	†	†	†	†	†	†	†	†	†	†
4	Irma	female	1	⌀	†	†	†	†	†	†	†	†	†	†	†	†	†	†	†
5	Maja	female	.	1	1	1	1	1	⌀?	†	†	†	†	†	†	†	†	†	†
6	Daniza	female	1	1	1	1	1	1	1	1	1	1	1	1	1	1	⌀	†	†
7	Joze	male	1	1	1	2	1	1	1	1	1	⌀?	†	†	†	†	†	†	†
8	Kirka	female	1	1	1	1	1	⌀?	†	†	†	†	†	†	†	†	†	†	†
9	Jurka	female	.	1	1	1	1	1	1	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ	Θ
10	Vida	female	.	1	⌀	†	†	†	†	†	†	†	†	†	†	†	†	†	†
11	KJ1	female	.	.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
12	KJ2	female	.	.	*	1	1	1	1	1	1	1	1	1	1	1	1	1	1
13	MJ1	male	.	.	.	*	⌀	†	†	†	†	†	†	†	†	†	†	†	†
14	MJ2	female	.	.	.	1	1	1	1	1	1	1	1	1	1	1	1	1	1
15	DJ1	female	.	.	.	.	1	2	1	1	1	1	1	⌀	†	†	†	†	†
16	DJ2	male	.	.	.	.	1	1	⌀?	†	†	†	†	†	†	†	†	†	†
17	DJ3	female	.	.	.	.	1	1	1	1	1	1	1	Θ	Θ	Θ	Θ	Θ	Θ
18	JJ1	male	.	.	.	.	1	1	⌀	†	†	†	†	†	†	†	†	†	†
19	JJ2	male	.	.	.	.	1	1	⌀?	†	†	†	†	†	†	†	†	†	†
20	MJ5	male	.	.	.	.	.	*	1	1	1	2	1	1	1	1	1	1	1
21	MJ3	male	.	.	.	.	.	1	⌀?	†	†	†	†	†	†	†	†	†	†
22	MJ4	male	.	.	.	.	.	1	1	1	1	1	1	1	1	1	1	1	1
23	BJ1	female	.	.	.	.	.	*	1	1	1	2	1	2	1	1	1	⌀	†
24	MJ2G1	male	.	.	.	.	.	.	*	1	1	1	1	1	1	1	1	1	1
25	JJ3	male	.	.	.	.	.	.	1	1	⌀	†	†	†	†	†	†	†	†
26	JJ4	female	.	.	.	.	.	.	1	1	1	1	1	1	1	1	1	1	1
27	JJ5	male	.	.	.	.	.	.	1	1	1	1	1	1	⌀	†	†	†	†
28	KJ2G1	female	.	.	.	.	.	.	1	1	⌀	†	†	†	†	†	†	†	†
29	KJ2G2	male	.	.	.	.	.	.	1	1	1	1	↗	↗	↗	↗	↗	↗	↗
30	DG1	female	.	.	.	.	.	.	⌀	†	†	†	†	†	†	†	†	†	†
31	DG2	male	.	.	.	.	.	.	1	1	1	1	2	1	1	⌀?	†	†	†
32	DG3	female	.	.	.	.	.	.	1	1	2	1	1	1	1	1	1	1	1
33	KJ1G1	female	.	.	.	.	.	.	1	1	1	1	2	⌀?	†	†	†	†	†
34	MJ2J1	male	.	.	.	.	.	.	1	⌀?	†	†	†	†	†	†	†	†	†
35	DJ1G1	male	.	.	.	.	.	.	.	1	2	2	2	2	⌀	†	†	†	†
36	DJ3G1	female	.	.	.	.	.	.	.	1	⌀?	†	†	†	†	†	†	†	†
37	M6	male	.	.	.	.	.	.	.	*	2	1	1	1	1	1	1	⌀	†
38	F1	female	.	.	.	.	.	.	.	.	⌀	†	†	†	†	†	†	†	†
39	F3	female	.	.	.	.	.	.	.	.	1	1	1	1	2	1	1	1	1
40	M2	male	.	.	.	.	.	.	.	.	1	1	1	1	1	⌀	†	†	†
41	M1	male	.	.	.	.	.	.	.	.	1	2	2	2	1	1	1	1	?
42	F4	female	.	.	.	.	.	.	.	.	1	2	2	1	1	1	1	1	1
43	F2	female	.	.	.	.	.	.	.	.	1	1	1	1	1	1	2	1	1
44	M3	male	.	.	.	.	.	.	.	.	1	1	1	1	1	1	1	1	?
45	M4	male	.	.	.	.	.	.	.	.	1	1	1	1	1	1	1	1	1
46	M7	male	.	.	.	.	.	.	.	.	.	1	2	1	1	1	1	1	1
47	M8	male	.	.	.	.	.	.	.	.	.	1	1	1	↗	↗	↗	↗	↗
48	F5	female	.	.	.	.	.	.	.	.	.	1	1	1	1	1	1	1	⌀
49	F6	female	.	.	.	.	.	.	.	.	.	.	⌀	†	†	†	†	†	†

N	Bear ID	Sex	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
50	F7	female	.	.	.	.	.	.	.	.	.	.	1	1	1	1	2	1	2
51	F8	female	.	.	.	.	.	.	.	.	.	.	1	1	1	1	1	2	2
52	F9	female	.	.	.	.	.	.	.	.	.	.	1	2	2	1	2	1	1
53	M9	male	.	.	.	.	.	.	.	.	.	.	1	1	1	ø?	†	†	†
54	F10	female	.	.	.	.	.	.	.	.	.	.	1	1	ø	†	†	†	†
55	M10	male	.	.	.	.	.	.	.	.	.	.	1	ø?	†	†	†	†	†
56	M12	male	.	.	.	.	.	.	.	.	.	.	*	1	ø	†	†	†	†
57	M13	male	.	.	.	.	.	.	.	.	.	.	*	1	1	ø	†	†	†
58	M14	male	.	.	.	.	.	.	.	.	.	.	*	1	ø	†	†	†	†
59	M11	male	.	.	.	.	.	.	.	.	.	.	.	1	1	ø?	†	†	†
60	F12	female	.	.	.	.	.	.	.	.	.	.	.	*	1	2	1	1	1
61	M15	male	.	.	.	.	.	.	.	.	.	.	.	.	1	1	1	2	1
62	F11	female	.	.	.	.	.	.	.	.	.	.	.	.	ø	†	†	†	†
63	F13	female	.	.	.	.	.	.	.	.	.	.	.	.	1	1	2	2	2
64	M16	male	.	.	.	.	.	.	.	.	.	.	.	.	1	ø?	†	†	†
65	M17	male	.	.	.	.	.	.	.	.	.	.	.	.	1	1	1	1	?
66	M18	male	.	.	.	.	.	.	.	.	.	.	.	.	1	1	1	1	1
67	M19	male	.	.	.	.	.	.	.	.	.	.	.	.	1	1	1	1	1
68	M20	male	.	.	.	.	.	.	.	.	.	.	.	.	1	1	ø	?	?
69	M21	male	.	.	.	.	.	.	.	.	.	.	.	.	1	1	1	1	ø
70	M22	male	.	.	.	.	.	.	.	.	.	.	.	.	1	1	1	1	1
71	F19	female	.	.	.	.	.	.	.	.	.	.	.	.	*	2	1	1	2
72	F14	female	.	.	.	.	.	.	.	.	.	.	.	.	*	1	1	1	2
73	F24	female	.	.	.	.	.	.	.	.	.	.	.	.	*	2	2	1	?
74	F16	female	.	.	.	.	.	.	.	.	.	.	.	.	*	1	1	2	1
75	F17	female	.	.	.	.	.	.	.	.	.	.	.	.	*	1	ø	?	?
76	M25	male	.	.	.	.	.	.	.	.	.	.	.	.	*	1	1	ø	†
77	F15	female	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	1	1
78	M26	male	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	ø	†
78	F18	female	.	.	.	.	.	.	.	.	.	.	.	.	.	1	2	1	1
80	M29	male	.	.	.	.	.	.	.	.	.	.	.	.	.	*	1	2	1
81	M27	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	ø	†	†
82	F20	female	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	1
83	M31	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	1
84	M30	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	?
85	F21	female	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	1
86	M32	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	ø
87	M33	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	ø	†
88	F22	female	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	ø	†
89	F23	female	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	?
90	F25	female	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1
91	M35	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1
92	M36	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1
93	M40	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*	1
94	M38	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	2
95	F28	female	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*	1
96	F26	female	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1
97	M39	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*	1

N	Bear ID	Sex	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
98	M41	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*	1
99	M42	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*	1
100	M43	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*	1
101	F27	female	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
102	M46	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
103	M47	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*

TABLE 3.6 – LIFE TABLE OF BEARS FROM THE CENTRAL ITALIAN ALPS POPULATION: THE FOUNDERS AND THEIR OFFSPRING (SEE LEGENDA IN MATERIALS AND METHODS)

Prog	Bear ID	Sex	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
1	Gen01		.	.	.	1	1	1	1	.	.	.	.	.	.	.	.	.
2	Gen02	male	1	.	.	1	1	1	.	.	.	.	.	.	.	.	.	.
3	Gen03	male	.	.	.	1	1	.	1	1	1	1	.	.	1	.	.	.
4	Gen04	male	.	.	.	1	.	.	1	1	1	1	1	1	1	1	.	.
5	Gen06	male	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.	.
6	Gen07	male	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.
7	Gen08	male	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
8	Gen09	male	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.
9	Gen10	male	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.
10	Gen11	male	.	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.
11	Gen12	male	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.
12	M5	male	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.
13	Gen14	male	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.
14	Gen15	male	.	.	.	.	.	.	.	.	.	1	1	1	.	1	.	.
15	Gen16	male	.	.	.	.	.	.	.	.	.	.	1	.	1	1	ø	†
16	Gen18	male	.	.	.	.	.	.	.	.	.	.	.	.	1	.	1	.
17	Gen19	male	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.
18	Gen20	male	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.
19	Gen21	male	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.
20	Gen22	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1
21	Gen23	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
22	Gen24	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
23	Gen25	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
*	KJ2G2	male	.	.	.	.	.	.	.	.	1	1	1	1	1	.	1	.
*	DG2	male	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.
*	M4	male	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
*	MJ2G1	male	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.
*	MJ4	male	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.
*	M8	male	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.

TABLE 3.7 - LIFE TABLE OF BEARS SAMPLED IN THE EASTERN ITALIAN ALPS

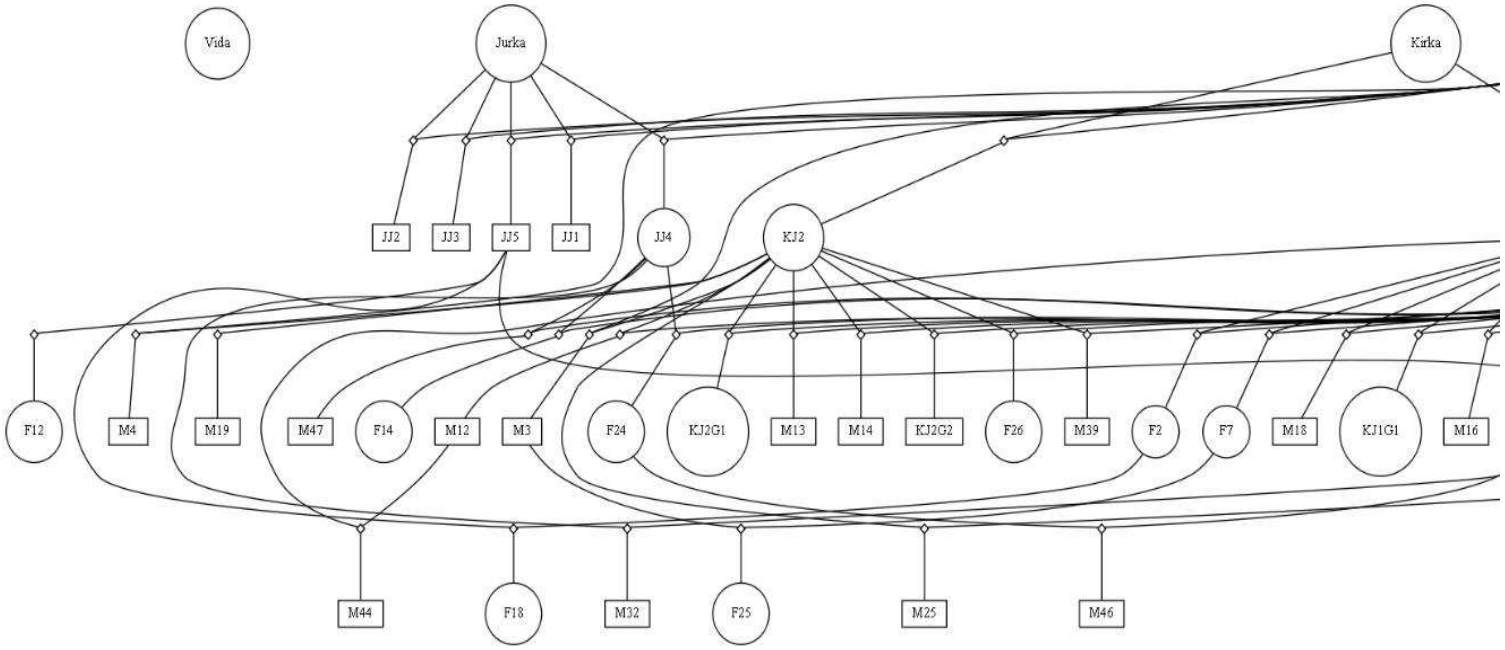
Parentage analysis and pedigree reconstruction

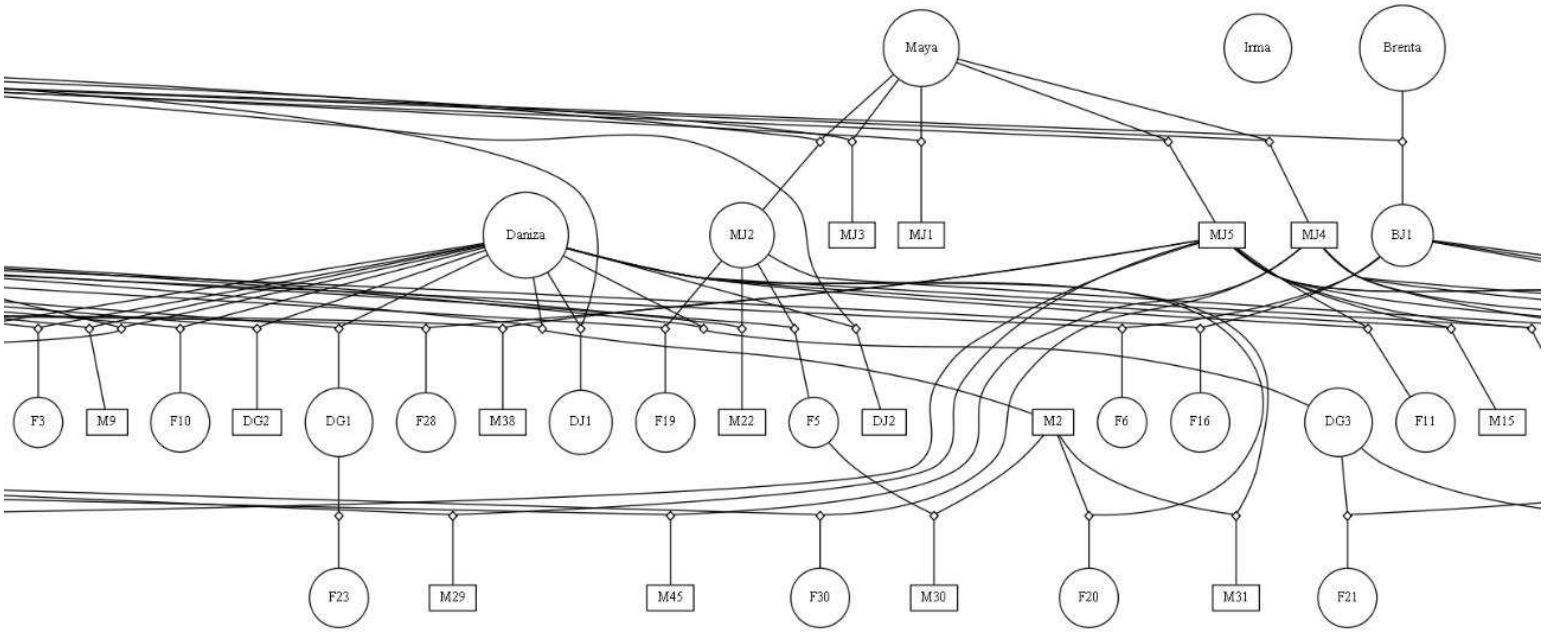
All but 6 individuals were identified as the progeny of founders and their descendant (Table 3.8). For the remaining 6 bears parentage relationships were uncertain: for bear M8 both software identified 1 MM (ADO) in the most probable trio; mother-assignments is missing for bear M10. Colony finds 4 MM with the most probable mother, FRANz finds 1 MM with a different mother; Colony and FRANz find two different fathers for M19, but Colony finds the most probable (M4) since 1MM is found by FRANz with M3. For M20, FRANz and Colony propose two different parents, but those are known to be wrong from field data. Incongruent results are also found for M21; Colony does not find any mother for M42, while Colony finds F13. The mean number of common loci typed among detected trio was 14.81, and mean probabilities of parentage assignment was 99.4%. No bears were found to be offspring of males dispersing from the Dinaric population. We found 24 bears of second generation (Gen II), 49 of the third generation (Gen III), and 14 of the fourth generation (Gen IV).

Offspring	Dam	Sire	Generation	Common loci typed	LOD	Probability	MM	Sig	Notes
Gasper	-	-	I	15	0.00E+00	1	0	<	
Brenta	-	-	1	15	0.00E+00	1	0	<	
Maya	-	-	1	15	0.00E+00	1	0	<	
Kirka	-	-	1	15	0.00E+00	1	0	<	
Jurka	-	-	1	15	0.00E+00	1	0	<	
Vida	-	-	1	10	0.00E+00	1	0	<	
Irma	-	-	1	10	0.00E+00	1	0	<	
Daniza	-	-	1	15	0.00E+00	1	0	<	
Joze	-	-	1	15	0.00E+00	1	0	<	
KJ1	Kirka	Joze	2	15	1.49E+01	0.9969	0	<	
KJ2	Kirka	Joze	2	15	1.26E+01	0.9864	0	<	
MJ1	Maya	Joze	2	10	1.19E+01	0.9282	0	<	
MJ2	Maya	Joze	2	15	1.65E+01	0.9968	0	<	
DJ1	Daniza	Joze	2	15	1.51E+01	0.9981	0	<	
DJ2	Daniza	Joze	2	15	1.83E+01	0.9978	0	<	
DJ3	Daniza	Joze	2	15	1.62E+01	0.9987	0	<	
JJ1	Jurka	Joze	2	15	1.75E+01	0.9918	0	<	
JJ2	Jurka	Joze	2	15	2.04E+01	0.9888	0	<	
MJ3	Maya	Joze	2	15	2.20E+01	0.9992	0	<	
MJ4	Maya	Joze	2	15	1.54E+01	0.9976	0	<	
DG1	Daniza	Gasper	2	15	1.23E+01	0.9119	1	<	
JJ3	Jurka	Joze	2	15	2.50E+01	0.9998	0	<	
JJ4	Jurka	Joze	2	15	1.76E+01	0.9627	0	<	
JJ5	Jurka	Joze	2	15	1.96E+01	0.9987	0	<	
KJ2G1	KJ2	Gasper	3	15	1.25E+01	0.9817	0	<	
KJ2G2	KJ2	Gasper	3	15	1.08E+01	0.9657	0	<	
MJ5	Maya	Joze	2	15	1.43E+01	0.9075	0	<	
DG2	Daniza	Gasper	2	15	1.79E+01	0.9955	0	<	
DG3	Daniza	Gasper	2	15	1.73E+01	0.9947	0	<	
KJ1G1	KJ1	Gasper	3	15	1.14E+01	0.9788	0	<	
BJ1	Brenta	Joze	2	15	2.21E+01	0.9997	0	<	
MJ2J1	MJ2	Joze	3	12	1.63E+01	0.9838	0	<	
DJ1G1	DJ1	Gasper	3	15	8.65E+00	0.8421	0	<	
DJ3G1	DJ3	Gasper	3	10	1.03E+01	0.9128	0	<	
MJ2G1	MJ2	Gasper	3	15	1.58E+01	0.9992	0	<	
F1	Daniza	Gasper	2	15	1.75E+01	0.9977	0	<	
F3	Daniza	Gasper	2	15	1.71E+01	0.9900	0	<	
M1	KJ1	Gasper	3	15	1.13E+01	0.9631	0	<	
F2	KJ1	Gasper	3	15	1.37E+01	0.9967	0	<	
M2	Daniza	Gasper	2	15	1.80E+01	0.9985	0	<	
M3	KJ2	Joze	3	15	1.40E+01	0.9505	0	<	
M4	KJ2	Joze	3	15	1.30E+01	0.9627	0	<	
F4	KJ1	Gasper	3	15	1.21E+01	0.9671	0	<	
M6	DJ3	Gasper	3	15	1.43E+01	0.9975	0	<	
M7	DJ3	Gasper	3	15	1.46E+01	0.9839	0	<	
M8*	DJ3?	Gasper?	?	15	9.80E+00	0.6769	1	<	1 ADO
F5	MJ2	Gasper	3	15	1.20E+01	0.9839	0	<	
F6	BJ1	Gasper	3	15	1.59E+01	0.9994	0	<	
F7	KJ1	Gasper	3	15	1.27E+01	0.9706	0	<	
F8	KJ1	Gasper	3	15	1.44E+01	0.9984	0	<	
F9	KJ1	Gasper	3	15	1.20E+01	0.6084	0	<	
M9	Daniza	Gasper	2	15	1.80E+01	0.9976	0	<	
F10	Daniza	Gasper	2	15	1.32E+01	0.8931	0	<	
M10*	?	Gasper	?	15	7.35E+00	0.9414	0	<	Mother not found
M11	DJ3	JJ5	3	15	1.79E+01	0.9985	0	<	
M12	KJ2	Gasper	3	15	1.26E+01	0.9790	0	<	
M13	KJ2	Gasper	3	15	1.21E+01	0.9884	0	<	

Offspring	Dam	Sire	Generation	Common loci typed	LOD	Probability	MM	Sig	Notes
M14	KJ2	Gasper	3	15	1.10E+01	0.9785	0	<	
M15	Daniza	MJ5	3	15	1.85E+01	0.9974	0	<	
F11	Daniza	MJ5	3	15	1.91E+01	0.9829	0	<	
F12	F2	JJ5	4	15	1.55E+01	0.9975	0	<	
F13	Daniza	MJ5	3	15	2.04E+01	0.9998	0	<	
M16	KJ1	Gasper	3	15	1.24E+01	0.9694	0	<	
M17	KJ1	Gasper	3	15	1.35E+01	0.9972	0	<	
M18	KJ1	Gasper	3	15	1.52E+01	0.9984	0	<	
M19*	KJ2?	M4?	?	15	1.95E+01	0.9940	0	<	Incongruent results
M20*	KJ1?	Gasper?	?	15	8.95E+00	0.9794	0	<	Incongruent results
M21*	F3?	M6?	?	15	3.99E+00	0.6809	0	<	Incongruent results
M22	MJ2	Gasper	3	15	1.44E+01	0.9952	0	<	
F14	JJ4	Gasper	3	15	1.06E+01	0.6805	0	<	
F15	F4	M6	4	15	1.23E+01	0.9181	0	<	
F16	BJ1	M2	3	15	1.58E+01	0.9925	0	<	
F17	BJ1	M2	3	15	1.83E+01	0.9999	0	<	
F18	F2	JJ5	4	15	1.73E+01	0.9804	0	<	
M25	KJ2	M6	4	15	1.07E+01	0.9567	0	<	
M26	F4	M6	4	15	1.21E+01	0.9332	0	<	
M27	F8	M1	4	15	1.51E+01	0.9935	0	<	
F19	MJ2	Gasper	3	15	1.18E+01	0.8733	0	<	
M29	F9	MJ5	4	15	1.48E+01	0.9771	0	<	
F20	Daniza	M2	3	15	2.13E+01	0.9985	0	<	
M30	F5	M2	3	15	1.46E+01	0.9989	0	<	
M31	Daniza	M2	3	15	2.08E+01	0.9715	0	<	
F21	DG3	MJ5	3	15	1.36E+01	0.9895	0	<	
M32	JJ4	MJ2G1	3	15	1.39E+01	0.9985	0	<	
M33	BJ1	MJ4	3	15	2.10E+01	0.9999	0	<	
F22	BJ1	MJ4	3	15	2.30E+01	1	0	<	
F23	F3	M1	4	15	3.17E+00	0.8744	0	<	
F24	JJ4	Gasper	3	15	1.30E+01	0.9877	0	<	
F25	F7	M3	4	15	1.57E+01	0.9977	0	<	
M35	F4	Gasper	4	15	1.71E+01	0.9957	0	<	
M36	F4	Gasper	4	15	1.75E+01	0.9963	0	<	
M38	KJ1	MJ5	3	15	1.64E+01	0.9984	0	<	
F26	KJ2	Gasper	3	15	1.30E+01	0.7831	0	<	
M39	KJ2	Gasper	3	15	1.50E+01	0.8781	0	<	
F27	DG3	MJ5	3	15	1.83E+01	0.9969	0	<	
M40	F4	Gasper	4	15	1.84E+01	0.9971	0	<	
M41	MJ2	MJ4	3	15	1.68E+01	0.9995	0	<	
M42*	F13?	MJ5	?	15	1.35E+01	0.9979	1	<	Incongruent results
F28	KJ1	MJ5	3	15	1.41E+01	0.9995	0	<	
M43	F9	MJ5	4	15	1.15E+01	0.8661	1	<	
M46	F24	MJ2G1	4	15	1.25E+01	0.8458	0	<	
M47	JJ4	Gasper	3	15	1.71E+01	0.5669	0	<	
				14.81		**0.9540**			

TABLE 3.8 – PARENTAGE RELATIONSHIPS AND PROBABILITIES OF ASSIGNMENTS AMONG EACH TRIO


Vida
Jurka
Kirka
JJ2
JJ3
JJ5
JJ1
JJ4
KJ2
F12
M4
M19
M47
F14
M12
M3
F24
KJ2G1
M13
M14
KJ2G2
F26
M39
F2
F7
M18
KJ1G1
M16
M44
F18
M32
F25
M25
M46


Maya
Irma
Brenta
Daniza
MJ2
MJ3
MJ1
MJ5
MJ4
BJ1
F3
M9
F10
DG2
DG1
F28
M38
DJ1
F19
M22
F5
DJ2
M2
F6
F16
DG3
F11
M15
F23
M29
M45
F30
M30
F20
M31
F21

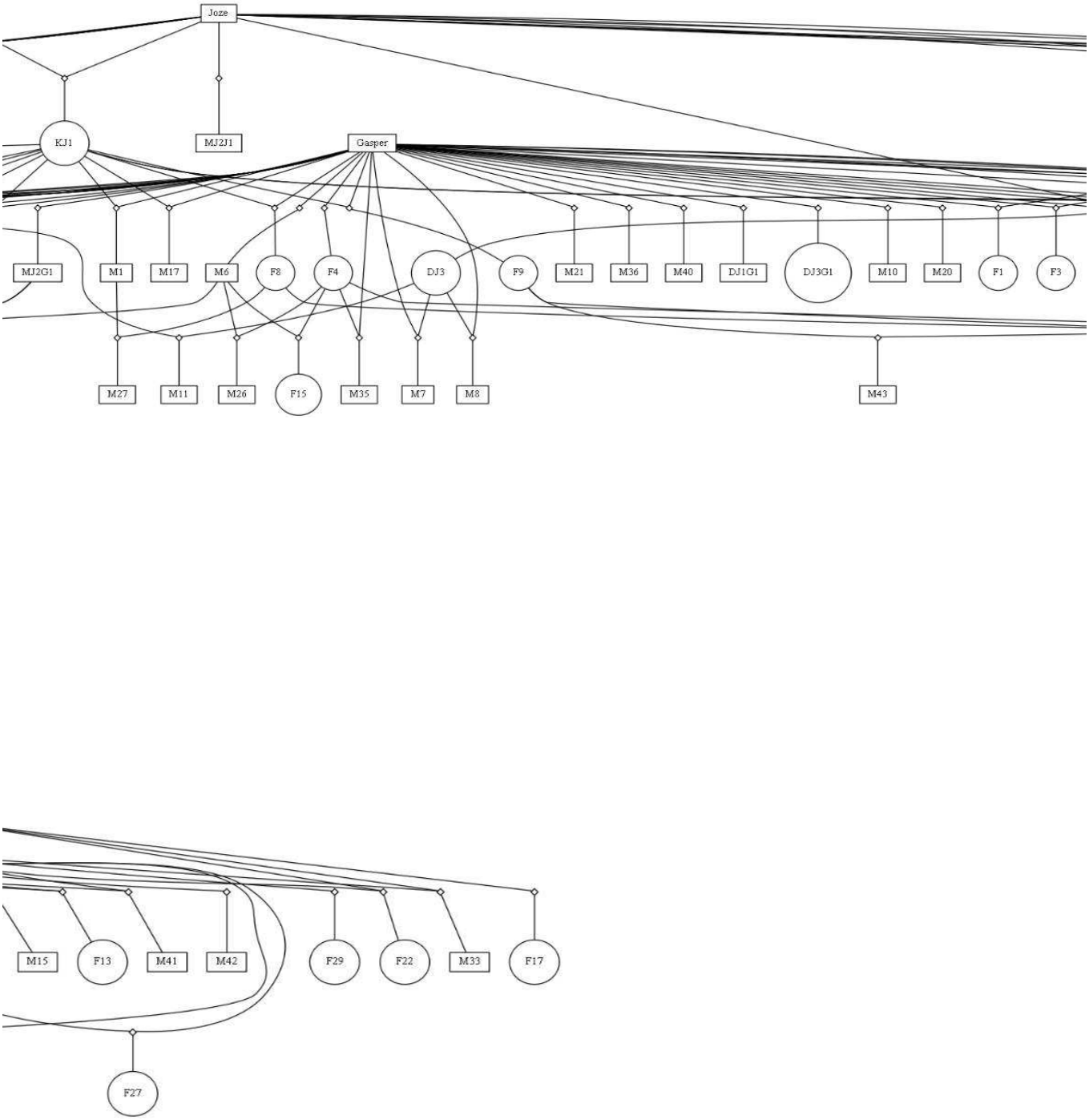


FIG. 3.4 - THE ALPINE BROWN BEAR POPULATION: PEDIGREE RECONSTRUCTION

Demographic data

The reintroduced population in central Italian Alps increased from the initial ten founders to a population size of ~45 individuals in 2016 (Fig. 3.5). A peak was registered in 2015, with a presence of at least 53 bears. These numbers are based on genetic sampling alone, thus they should be considered as a minimum count: it is likely that bears born in the last few years escaped the genetic sampling, and will be probably sampled in the future. However, it was evident that the population has been exponentially growing since the reintroduction program started. Conversely, it was impossible to describe a demographic trend among bears sampled in eastern Italy since this is part of the larger Dinaric population. However, the occurrence of bears was highly variable from one year to the next (from 1 to 7 bears) in eastern Italy, but a slight increase over time was manifest. The turnover was high especially for bears that have been sampled in this area since 2006, meaning that bears frequently returned to the territory of origin, often moving back and forth the Slovenian territory of origin.

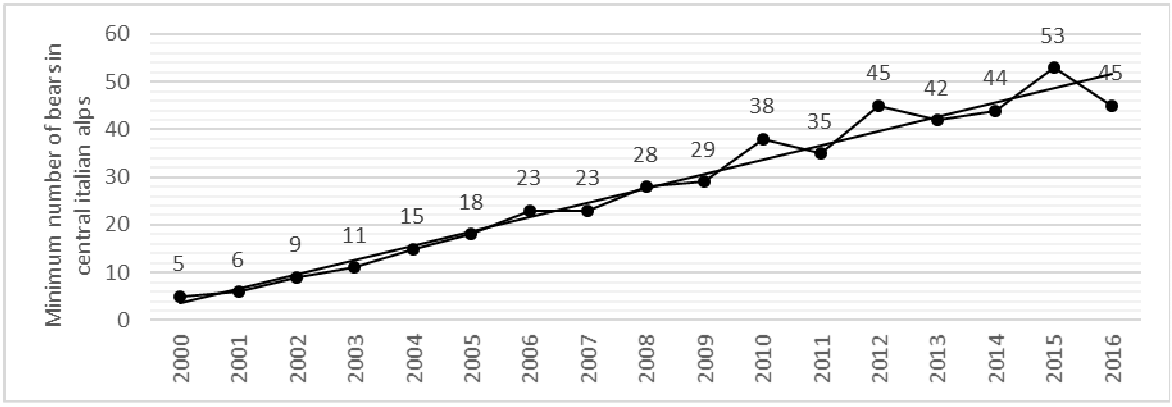


FIG. 3.5 – MINIMUM NUMBER OF BEARS IN CENTRAL ITALIAN ALPS BETWEEN 2000 AND 2016

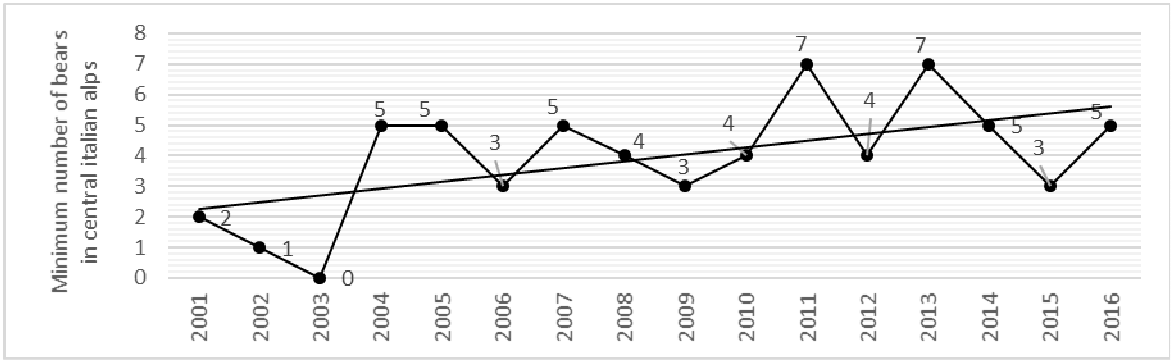


FIG. 3.6 – MINIMUM NUMBER OF BEARS IN EASTERN ITALY BETWEEN 2001 AND 2016

The sex ratio ranged from 1 female to 0.2 males in 2002, when only one male founder was released and still alive, to 1:1. An approximate equal sex ratio was registered in many years starting from 2008, while it was skewed towards females until 2007 (fig 3.7).

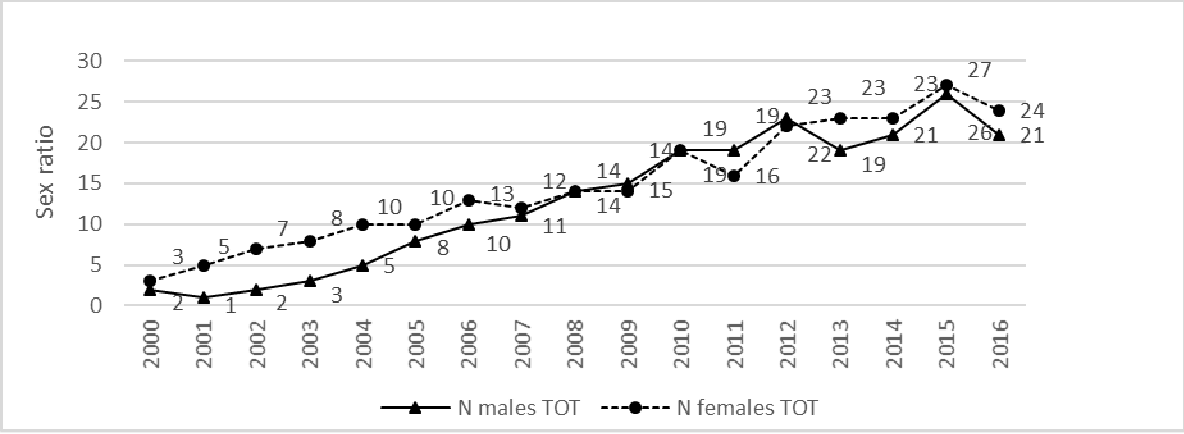


FIG. 3.7 –SEX RATIO OF BEARS IN CENTRAL ITALIAN ALPS BETWEEN 2000 AND 2016

The number of cubs, juveniles, and adults is reported (fig. 3.8): number of juveniles and adults has been growing over years. Reproductivity success was high: the first reproductive event was registered in 2002, and bears have been continued to reproduce until 2016 (48 litters) for a total of 53 male cubs and 40 female cubs (93 cubs)(table 3.9). The number of cubs per year greatly fluctuated, with a maximum of 12 cubs born in 2012, and a minimum of 2 cubs in 2011 (without considering years 2002 and 2003, when only founders were able to reproduce). Since 2002, 21 females (5 founders and 16 born after the reintroduction) reproduced with 11 males (2 founders and 9 born after the reintroduction), with an average of 2.4 litters per female and 4.3 cubs (Table 3.10). Daniza produced the highest number of litters (6), followed by KJ1 and KJ2 (both 5 litters). Average values were calculated excluding cubs with uncertain kinship (7 cubs), meaning that these numbers are slightly lower than the real values. Females had their first litter at different ages, but the 6 youngest reproduced at the age of 3 for the first time. One female (DG3) reproduced at the age of 9. For females that had multiple litters, the interbirth interval was always 2 years, with only one exception (BJ1, three years once). Average fecundity of females was 1.5 for the first litter and increased to 1.9 and 2.1 with the second and third litter. For the few females that had a fourth and fifth litter, average fecundity was 1.7 and 2.3 respectively.

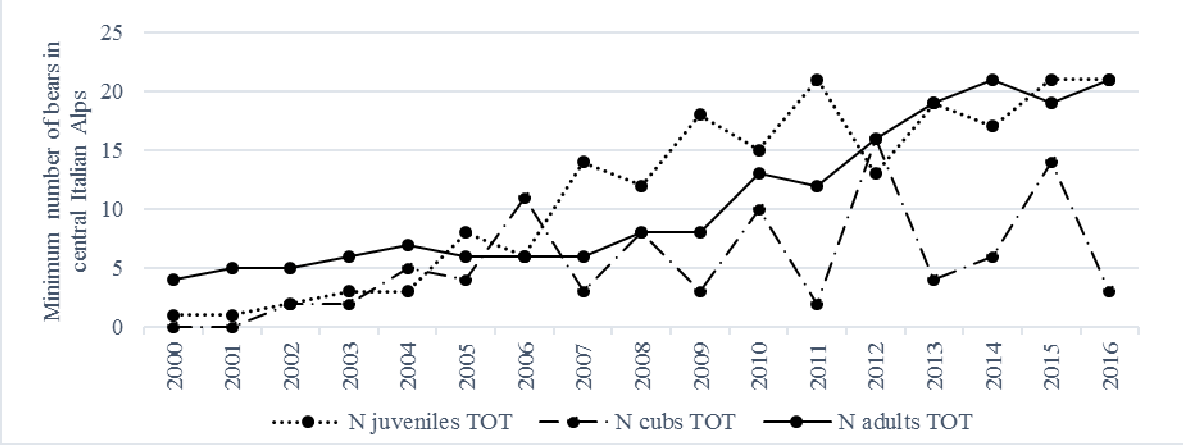
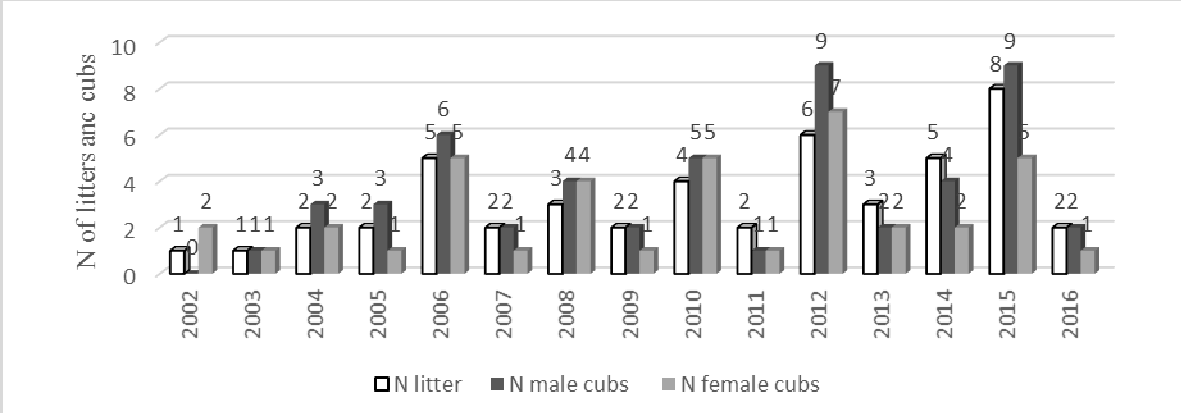


FIG. 3.8 – AGE STRUCTURE OF BEARS IN CENTRAL ITALIAN ALPS BETWEEN 2000 AND 2016



	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	TOT
N of litters	1	1	2	2	5	2	3	2	4	2	6	3	5	8	2	**48**
N male cubs	0	1	3	3	6	2	4	2	5	1	9	2	4	9	2	**53**
N female cubs	2	1	2	1	5	1	4	1	5	1	7	2	2	5	1	**40**
TOT cubs	2	2	5	4	11	3	8	3	10	2	16	4	6	14	3	**93**

TABLE 3.9 AND FIG 3.9 – REPRODUCTIVE SUCCESS OF BEARS IN CENTRAL ITALIAN ALPS BETWEEN 2000 AND 2016

Reproducing females	N cubs	N litters	Litter 1		Litter 2		Litter 3		Litter 4		Litter 5		Litter 6	
			Year	N cubs	Year	N cubs	Year	N cubs	Year	N cubs	Year	N cubs	Year	N cubs
BJ1	**5**	**3**	2010	1	2012	2	2015	2						
Brenta	**1**	**1**	2005	1										
Daniza	**16**	**6**	2004	3	2006	3	2008	3	2010	2	2012	3	2014	2
DG3	**2**	**2**	2014	1	2016	1								
DJ1	**1**	**1**	2007	1										
DJ3	**4**	**3**	2007	2	2009	1	2011	1						
F2	**2**	**2**	2011	1	2013	1								
F3	**1**	**1**	2015	1										
F4	**5**	**2**	2013	2	2015	3								
F5	**1**	**1**	2014	1										
F7	**1**	**1**	2015	1										
F8	**1**	**1**	2014	1										
F9	**2**	**2**	2013	1	2015	1								
JJ4	**4**	**3**	2012	2	2014	1	2016	1						
Jurka	**5**	**2**	2004	2	2006	3								
Kirka	**2**	**1**	2002	2										
KJ1	**12**	**5**	2006	1	2008	3	2010	3	2012	3	2015	2		
KJ2	**10**	**5**	2006	2	2008	2	2010	3	2012	1	2015	2		
Maja	**5**	**2**	2003	2	2005	3								
MJ2	**6**	**4**	2006	2	2009	1	2012	2	2015	1				
Mean	**4.3**	**2.4**		**1.5**		**1.9**		**2.1**		**1.7**		**2.3**		

TABLE 3.10 – FECUNDITY OF FEMALE BEARS IN CENTRAL ITALIAN ALPS BETWEEN 2000 AND 2016

Survival rates and mortality

Mean survival rates registered from 2000 to 2016 was 0.86 for cubs, 0.79 for juveniles and 0.89 for adults. No significant differences among age groups are evident. However, lowest survival rates were registered for cubs between 2003 and 2004 (0.50%) and between 2007 and 2008 (0.67%). Low survival rates were registered also for juveniles and between 2005 and 2006 (0.67%). Overall, survival rates increased over the years for all age groups (fig 3.10).

Among all 102 bears, 34 are still alive at the end of 2017, two (Jurka and DJ3) were moved to captivity in 2007 and 2011 respectively, because they had a problematic attitude toward humans. 3 bears (KJ2G2, M4and M8) emigrated to the eastern portion of the study area and were never sampled again in central Italian Alps. 17 “disappeared”, but their disappearance is too recent to establish their fate. 32 bears were found dead, while 15 bears are assumed to be dead since carcasses were not found but they have not been sampled for at least to consecutive years. Several are the causes of death: two died under an avalanche, 3 (Daniza, KJ2G1, and JJ5) died during the capture or following negative consequences due to the capture, 4 were found dead after collisions with cars or trains, 4 have been culled in neighboring countries after dispersal, 2 were poached, 1 was probably poached, 3 were poisoned. One bear died for an intraspecific aggression: a male killed BJ1 and its two cubs (Davoli et al 2018). 8 died for natural causes, while two deaths have unknown causes. Given these numbers, 16 out of 32 deaths (50.0%) were human-induced. The oldest bear (Daniza) died at the age of 19, while other three bears (KJ1, Joze, and Gasper) died at the age of 15. Eight cubs were found dead, while 19 died before reaching sexual maturity (age 1-3 for females and 1-4 for males).

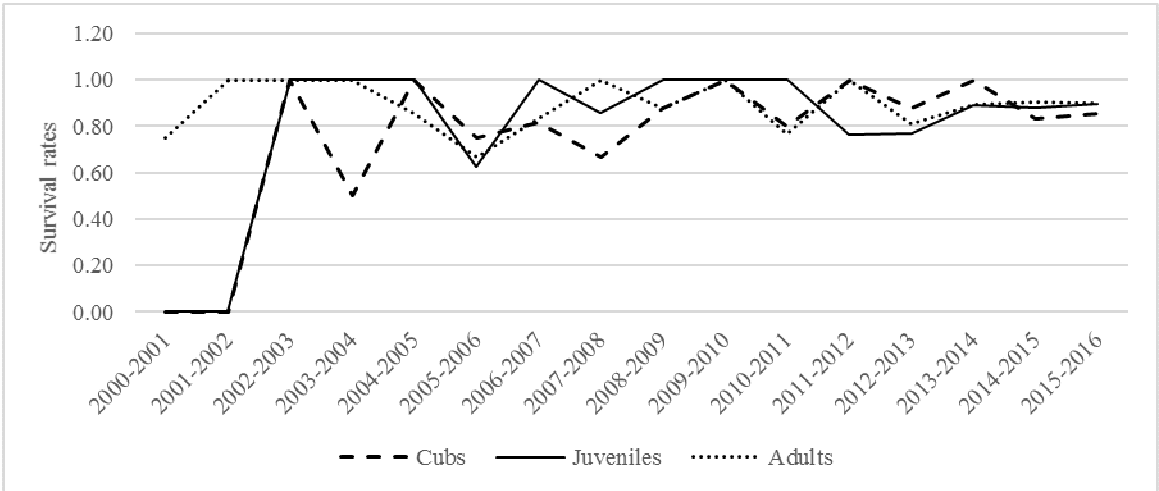


FIG. 3. 10 - SURVIVAL RATES OF CUBS, JUVENILES AND ADULTS IN CENTRAL ITALIAN ALPS BETWEEN 2000 AND 201

Prog	Bear ID	Sex	Year of birth	Fate	Cause of death	Year of death	Age of death	Movements
1	**Masun**	male	1996	Hyp Dead	-	2001?	5	Only core area
2	**Gasper**	male	1999	Dead	Unknown	2014	15	Only core area
3	**Brenta**	female	1999	Dead	Avalanche	2006	7	Only core area
4	**Irma**	female	1995	Dead	Avalanche	2001	6	Only core area
5	**Maja**	female	1997	Hyp Dead	-	2006?	9	Only core area
6	**Daniza**	female	1995	Dead	Capture	2014	19	Only core area
7	**Joze**	male	1994	Hyp Dead	-	2009?	15	Only core area
8	**Kirka**	female	1996	Hyp Dead	-	2005?	9	Only core area
9	**Jurka**	female	1997	Captivated	-			Only core area
10	**Vida**	female	1998	Dead	Unknown	2002	4	Only core area
11	**KJ1**	female	2002	Alive	-			Only core area
12	**KJ2**	female	2002	Dead	Culled	2017	15	Only core area
13	**MJ1**	male	2003	Dead	Natural	2003	0	Only core area
14	**MJ2**	female	2003	Disappeared	-			Only core area
15	**DJ1**	female	2004	Dead	Unknown	2011	7	Only core area
16	**DJ2**	male	2004	Hyp Dead	-	2006?	2	Only core area
17	**DJ3**	female	2004	Captivated	-			Only core area
18	**JJ1**	male	2004	Dead	Culled	2006	2	Dispersal central Alps
19	**JJ2**	male	2004	Hyp Dead		2006?	2	Dispersal central Alps
20	**MJ3**	male	2005	Hyp Dead	-	2006?	1	Only core area
21	**MJ4**	male	2005	Alive	-			Dispersal eastern Alps
22	**DG1**	female	2006	Dead	Unknown	2006	0	Only core area
23	**JJ3**	male	2006	Dead	Culled	2008	2	Dispersal central Alps
24	**JJ4**	female	2006	Alive	-			Only core area
25	**JJ5**	male	2006	Dead	Capture	2012	6	Dispersal central Alps
26	**KJ2G1**	female	2006	Dead	Capture	2008	2	Only core area
27	**KJ2G2**	male	2006	Emigrated				Dispersal eastern Alps
28	**MJ5**	male	2005	Alive	-			Dispersal central Alps
29	**DG2**	male	2006	Hyp Dead	-	2013?	7	Dispersal eastern Alps
30	**DG3**	female	2006	Alive	-			Only core area
31	**KJ1G1**	female	2006	Hyp Dead	-	2011?	5	Only core area
32	**BJ1**	female	2005	Dead	Intraspecific	2015	10	Only core area
33	**MJ2J1**	male	2006	Hyp Dead	-	2007?	1	Only core area
34	**DJ1G1**	male	2007	Dead	Unknown	2012	5	Dispersal central Alps
35	**DJ3G1**	female	2007	Hyp Dead	-	2008?	1	Only core area
36	**MJ2G1**	male	2006	Alive	-			Dispersal eastern Alps
37	**F1**	female	2008	Dead	Collision	2008	0	Only core area
38	**F3**	female	2008	Alive	-			Only core area
39	**M1**	male	2008	Disappeared	-			Dispersal central Alps
40	**F2**	female	2008	Alive	-			Only core area
41	**M2**	male	2008	Dead	Poached	2013	5	Dispersal central Alps
42	**M3**	male	2008	Disappeared	-			Dispersal central Alps
43	**M4**	male	2008	Emigrated				Dispersal eastern Alps
44	**F4**	female	2008	Alive	-			Only core area
45	**M6**	male	2007	Dead	Poisoned	2015	8	Dispersal central Alps
46	**M7**	male	2009	Alive	-			Dispersal central Alps
47	**M8**	male	2009	Emigrated				Dispersal eastern Alps
48	**F5**	female	2009	Dead	Poisoned	2016	7	Only core area
49	**F6**	female	2010	Dead	Infanticide?	2010	0	Only core area
50	**F7**	female	2010	Alive	-			Only core area
51	**F8**	female	2010	Alive	-			Only core area
52	**F9**	female	2010	Alive	-			Only core area
53	**M9**	male	2010	Hyp Dead	-	2013?	3	Dispersal central Alps
54	**F10**	female	2010	Dead	Unknown	2012	2	Only core area
55	**M10**	male	2010	Hyp Dead		2011?	1	Only core area
56	**M11**	male	2011	Hyp Dead	Hyp poached	2013?	2	Only core area
57	**M12**	male	2010	Dead	Collision	2012	2	Dispersal central Alps
58	**M13**	male	2010	Dead	Culled	2013	3	Dispersal central Alps
59	**M14**	male	2010	Dead	Collision	2012	2	Dispersal central Alps
60	**M15**	male	2012	Alive	-			Only core area

Prog	Bear ID	Sex	Year of birth	Fate	Cause of death	Year of death	Age of death	Movements
61	**F11**	female	2012	Dead	Natural	2012	0	Only core area
62	**F12**	female	2011	Alive	-			Only core area
63	**F13**	female	2012	Alive	-			Only core area
64	**M16**	male	2012	Hyp Dead	-	2013?	1	Only core area
65	**M17**	male	2012	Disappeared	-			Dispersal central Alps
66	**M18**	male	2012	Alive	-			Dispersal central Alps
67	**M19**	male	2012	Alive	-			Dispersal eastern Alps
68	**M20**	male	2012	Disappeared	-			Dispersal central Alps
69	**M21**	male	2012	Dead	Poisoned	2016	4	Only core area
70	**M22**	male	2012	Alive	-			Dispersal central Alps
71	**F14**	female	2012	Alive	-			Only core area
72	**F15**	female	2013	Alive	-			Only core area
73	**F16**	female	2012	Alive	-			Only core area
74	**F17**	female	2012	Disappeared	-			Only core area
75	**F18**	female	2013	Disappeared	-			Only core area
76	**M25**	male	2012	Dead	Poached	2015	3	Dispersal central Alps
77	**M26**	male	2013	Dead	Unknown	2015	2	Only core area
78	**M27**	male	2014	Dead	Natural	2014	0	Only core area
79	**F19**	female	2012	Alive	-			Only core area
80	**M29**	male	2013	Alive	-			Dispersal central Alps
81	**F20**	female	2014	Alive	-			Only core area
82	**M30**	male	2014	Disappeared	-			Only core area
83	**M31**	male	2014	Alive	-			Dispersal central Alps
84	**F21**	female	2014	Disappeared	-			Only core area
85	**M32**	male	2014	Dead	Collision	2016	2	Dispersal central Alps
86	**M33**	male	2015	Dead	Infanticide	2015	0	Only core area
87	**F22**	female	2015	Dead	Infanticide	2015	0	Only core area
88	**F23**	female	2015	Disappeared	-			Only core area
89	**F24**	female	2012	Alive	-			Only core area
90	**F25**	female	2015	Disappeared	-			Only core area
91	**M35**	male	2015	Disappeared	-			Only core area
92	**M36**	male	2015	Disappeared	-			Only core area
93	**M38**	male	2015	Alive	-			Only core area
94	**F26**	female	2015	Alive	-			Only core area
95	**M39**	male	2015	Disappeared	-			Only core area
96	**F27**	female	2016	Disappeared	-			Only core area
97	**M40**	male	2015	Disappeared	-			Only core area
98	**M41**	male	2015	Disappeared	-			Only core area
99	**M42**	male	2015	Alive	-			Only core area
100	**F28**	female	2015	Alive	-			Only core area
101	**M43**	male	2015	Alive	-			Only core area
102	**M46**	male	2016	Alive	-			Dispersal central Alps
103	**M47**	male	2016	Alive	-			Dispersal central Alps

TABLE 3.11 – FATE OF BEARS IN CENTRAL ITALIAN ALPS IN 2016

Census population size

Census estimates of the reintroduced population in central Italian Alps were low at the end of 2016 (Table 3.12 and Fig. 3.9), ranging from 43 to 48 bears using the ECM or the TIRMPart approach, respectively. Estimates of population size slightly varied among different methods, and higher differences among estimated values and minimum numbers of bears genetically identified are reported starting from 2010.

	ECM			TIRM			TIRMPart			Nmin
	Pop.size	CI (2,5%)	CI (97,5%)	Pop.size	CI (2,5%)	CI (97,5%)	Pop.size	CI (2,5%)	CI (97,5%)	
2003	14	14	14	14	14	15	15	15,00	43,00	**11**
2004	18	18	18	18	18	18	17	17,00	18,00	**15**
2005	22	22	22	22	22	22	17	17,00	17,00	**18**
2006	27	27	27	28	27	29	29	28,00	29,00	**23**
2007	28	28	28	28	28	28	28	28,00	30,00	**23**
2008	32	32	32	32	32	33	32	32,00	33,00	**28**
2009	29	29	29	29	29	29	27	25,00	29,00	**29**
2010	36	36	36	36	36	36	31	31,00	33,00	**38**
2011	36	36	36	37	33	37	36	32,00	37,00	**35**
2012	44	44	44	44	44	44	45	44,00	48,00	**45**
2013	47	47	47	47	47	47	45	41,00	49,00	**42**
2014	44	44	44	47	47	53	51	51,00	57,00	**44**
2015	45	45	45	45	45	47	55	55,00	59,00	**53**
2016	43	43	43	43	43	43	48	48,00	61,00	**45**

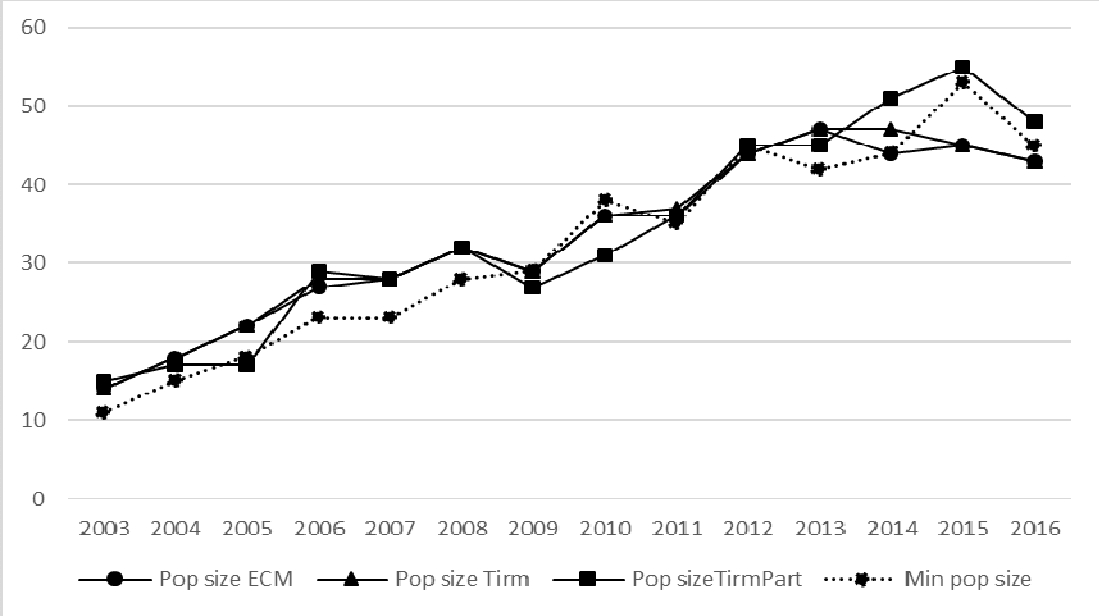


TABLE 3.12 AND FIG. 3.11 - ESTIMATION OF POPULATION SIZE FROM NON-INVASIVE SAMPLING BY YEARS USING THREE DIFFERENT MODELS: EQUAL CAPTURE MODEL (ECM), TWO-INNATE RATES MODEL (TIRM) AND TWO-INNATE RATES MODEL AFTER PARTITIONING DATA (TIRMPART)

Geographic distribution

All female bears were sampled in the surroundings of the translocation area (TN-AD), and no dispersal events were observed. Conversely, many males dispersed all over the study area. (Fig 3.10). First Dinaric bears where sampled in FVG in 2011 (geographic coordinates where not collected), wheater first males from the central Italian Alps, all offspring of translocated bears, started dispersing towards west (LOMB) and east (VEN) in 2007, with the exception of one male bear that dispersed to LOMB in 2003 (26 male bears in total). Bear occurrence has become stable in LOMB and VEN since 2007 (Fig 3.11). In addition, starting from 2011, 7 males born in central Italian Alps were sampled at greater distances, in FVG, where also males dispersing from the Dinaric population where present at that time. Bears were sampled in the neighbouring countries: 3 bears were sampled in Austria between 2005 and 2016; 4

bears were sampled in Switzerland between 2005 and 2007 and 1 bear was sampled in Germany in 2006 (not represented in fig 3.11). 26 male bears, including founders, did not dispersed from the translocation area.

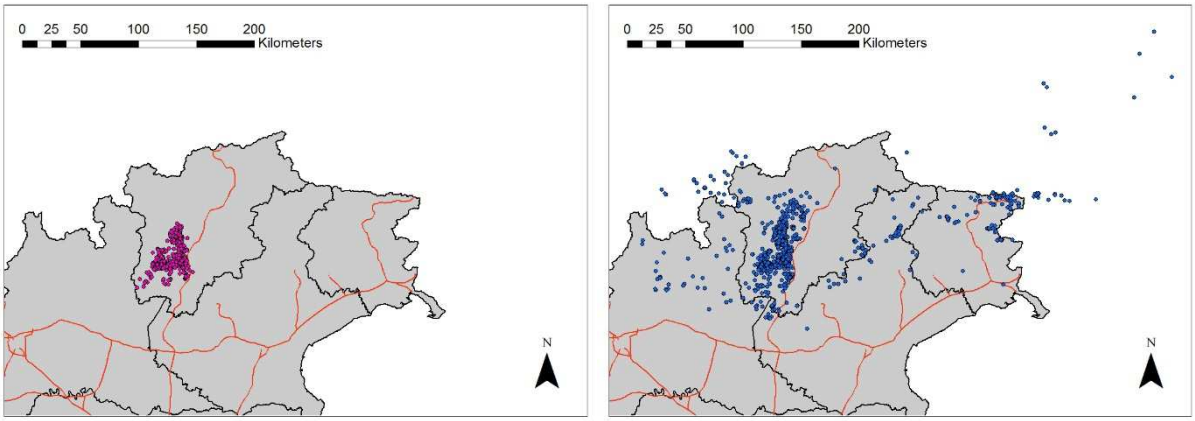
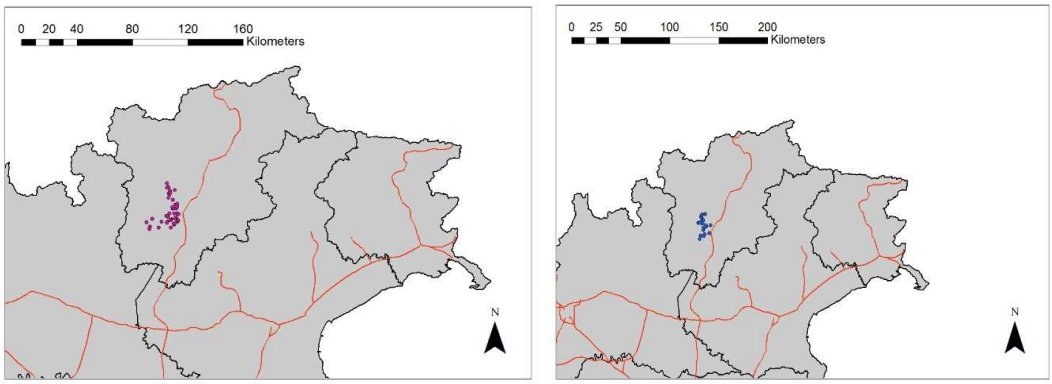
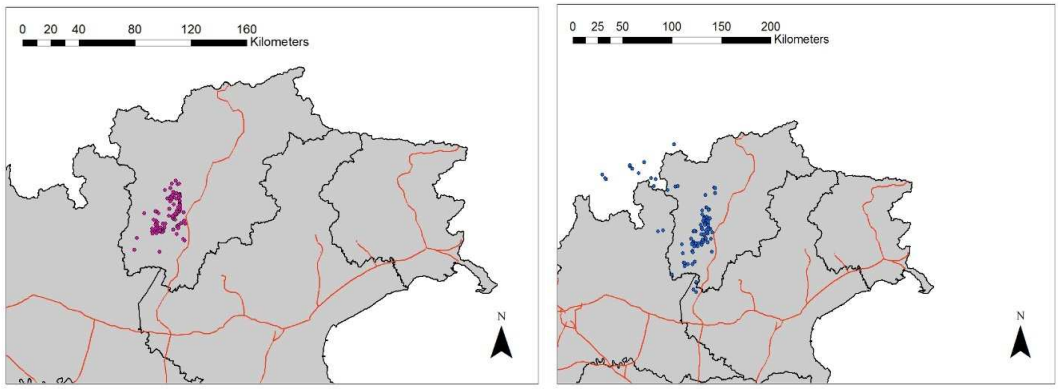


FIGURE 3.12 – FEMALES (LEFT) AND MALES BEAR OCCUREANCE BETWEEN 2000 AND 2016

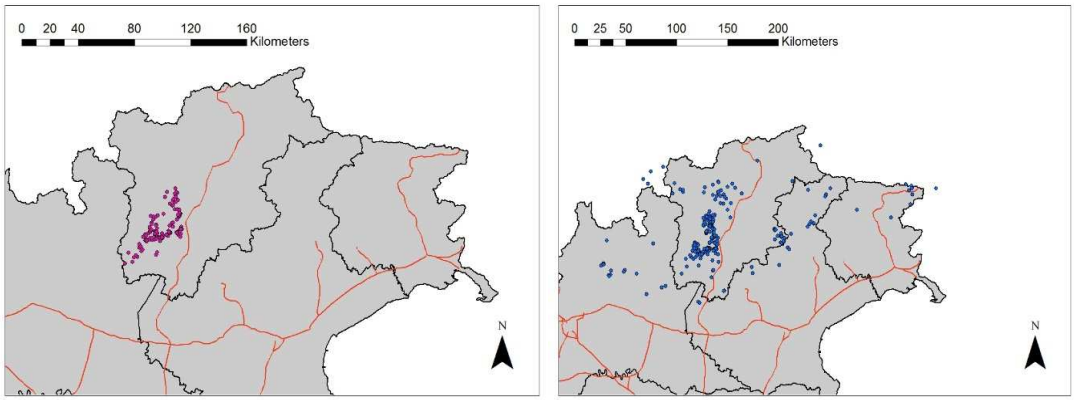
a)



b)



c)



d)

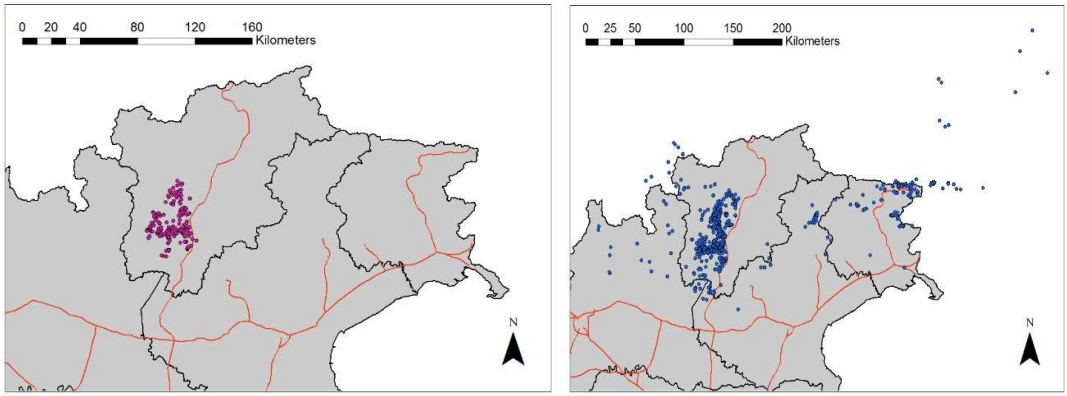


FIGURE 3.13 – FEMALES (LEFT) AND MALE (RIGHT) BEAR OCCURENCE OVER YEARS: A) 2000-2004; B)2005-2007; C)2008-2010; D)2011-2016

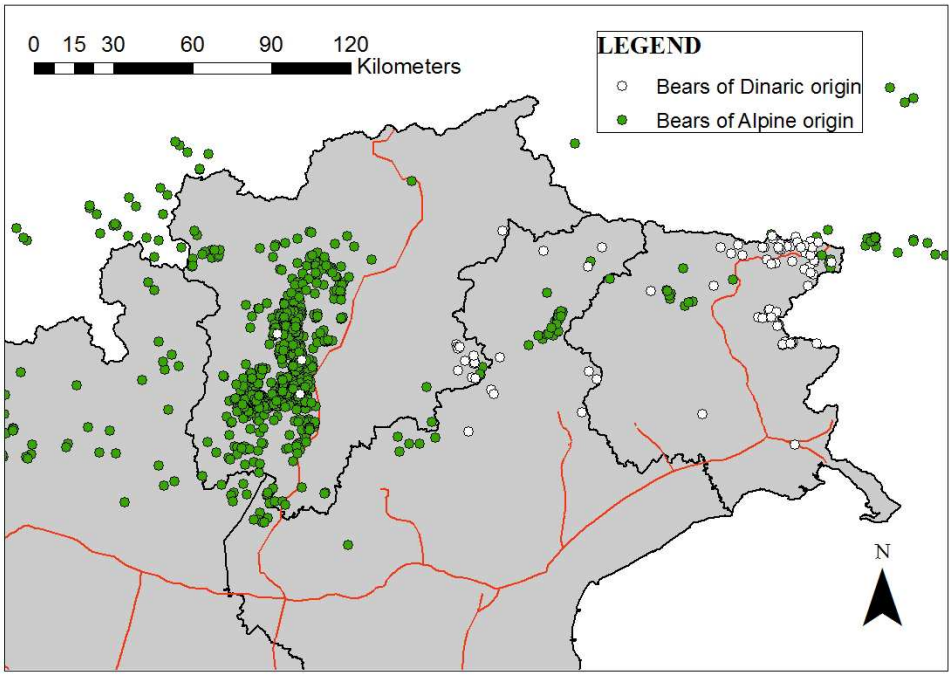


FIG. 3.14 – OCCURRENCE OF BEARS OF DINARIC ORIGIN (IN WHITE) AND BEARS OF ALPINE ORIGIN (IN GREEN)

Genetic diversity, inbreeding and effective population size

Changes in allele frequencies per locus over generation are summarized in table 3.14, while values of genetic diversity measures (mean and standard errors) are indicated in table 3.15. Genetic diversity is relatively high (He from 0.702 in the founders, to 0.618 in generation IV, fig. 3.15). However, number of alleles per locus (Na), effective number of alleles (Ne), Shannon's information index (I), expected unbiased (uHe) heterozygosity, expected heterozygosity (He) and fixation index (F) showed a declining trend over generations (tables 3.14 and fig 3.10). Private alleles are found only among founders (fig. 3.15).

Locus	Allele/n	Gen_I	Gen_II	Gen_III	Gen_IV	Locus	Allele/n	Gen_I	Gen_II	Gen_III	Gen_IV
G10M	**N**	**9.00**	**24.00**	**49.00**	**14.00**	**Mu50**	**N**	**9.00**	**24.00**	**49.00**	**14.00**
	111	0.22	0.23	0.31	0.46		84	0.22	0.15	0.36	0.39
	117	0.06	0.25	0.22	0.11		98	0.28	0.33	0.20	0.21
	119	0.11	0.17	0.07	0.00		100	0.11	0.00	0.00	0.04
	121	0.06	0.00	0.00	0.00		102	0.11	0.08	0.07	0.07
	123	0.39	0.33	0.39	0.43		104	0.17	0.31	0.19	0.07
	125	0.17	0.02	0.01	0.00		106	0.11	0.13	0.17	0.21
G10P	**N**	**9.00**	**24.00**	**49.00**	**14.00**	**Mu59**	**N**	**9.00**	**24.00**	**49.00**	**14.00**
	151	0.22	0.25	0.20	0.11		99	0.06	0.00	0.00	0.00
	165	0.11	0.17	0.31	0.39		101	0.17	0.35	0.44	0.64
	167	0.06	0.02	0.06	0.07		103	0.17	0.06	0.12	0.14
	169	0.33	0.21	0.21	0.32		111	0.11	0.19	0.08	0.00
	171	0.22	0.35	0.21	0.11		113	0.06	0.13	0.08	0.00
	173	0.06	0.00	0.00	0.00		117	0.06	0.00	0.00	0.00
G10X	**N**	**9.00**	**24.00**	**49.00**	**14.00**		119	0.06	0.00	0.00	0.00
	131	0.11	0.02	0.09	0.18		121	0.11	0.00	0.00	0.00
	133	0.11	0.15	0.07	0.11		123	0.22	0.27	0.28	0.21
	139	0.44	0.27	0.37	0.29	**G10C**	**N**	**8.00**	**23.00**	**48.00**	**14.00**
	143	0.28	0.46	0.36	0.14		94	0.19	0.41	0.35	0.29
	153	0.06	0.10	0.11	0.29		102	0.13	0.00	0.00	0.00
G1D	**N**	**9.00**	**24.00**	**49.00**	**14.00**		104	0.13	0.07	0.04	0.00
	102	0.17	0.27	0.27	0.25		106	0.44	0.48	0.60	0.68
	106	0.22	0.15	0.18	0.14		110	0.13	0.04	0.00	0.04
	108	0.22	0.35	0.46	0.54	**G10H**	**N**	**8.00**	**23.00**	**48.00**	**14.00**
	110	0.11	0.06	0.00	0.04		233	0.19	0.09	0.07	0.14
	114	0.06	0.06	0.02	0.00		251	0.00	0.02	0.00	0.07
	116	0.22	0.10	0.07	0.04		253	0.81	0.89	0.93	0.79
Mu11	**N**	**9.00**	**24.00**	**49.00**	**14.00**	**G10L**	**N**	**8.00**	**23.00**	**47.00**	**14.00**
	78	0.50	0.40	0.27	0.29		151	0.56	0.48	0.60	0.68
	80	0.06	0.08	0.16	0.25		153	0.25	0.28	0.17	0.07
	86	0.17	0.25	0.38	0.32		159	0.19	0.24	0.23	0.25
	88	0.17	0.19	0.12	0.14	**Mu09**	**N**	**8.00**	**23.00**	**47.00**	**14.00**
	90	0.11	0.08	0.07	0.00		177	0.06	0.11	0.02	0.07
Mu15	**N**	**9.00**	**24.00**	**49.00**	**14.00**		185	0.19	0.07	0.11	0.07
	132	0.22	0.21	0.03	0.07		193	0.19	0.24	0.31	0.21
	136	0.17	0.27	0.24	0.43		195	0.38	0.46	0.35	0.32
	138	0.06	0.02	0.05	0.04		197	0.06	0.07	0.05	0.00
	142	0.33	0.29	0.27	0.07		203	0.13	0.07	0.16	0.32
	146	0.22	0.21	0.41	0.39	**Mu10**	**N**	**8.00**	**23.00**	**47.00**	**14.00**
Mu23	**N**	**9.00**	**24.00**	**49.00**	**14.00**		118	0.44	0.61	0.61	0.57
	118	0.22	0.23	0.44	0.36		128	0.38	0.33	0.19	0.18
	120	0.11	0.19	0.26	0.21		130	0.19	0.07	0.20	0.25
	122	0.06	0.02	0.02	0.00	**cxx20**	**N**	**9.00**	**24.00**	**49.00**	**14.00**
	124	0.39	0.46	0.26	0.39		118	0.17	0.23	0.19	0.14
	126	0.06	0.00	0.00	0.00		120	0.17	0.27	0.11	0.04
	128	0.17	0.10	0.03	0.04		130	0.11	0.23	0.34	0.39
							134	0.56	0.27	0.36	0.43

TABLE 3.14 – ALLELE FREQUENCIES AND SAMPLE SIZE PER LOCUS AND GENERATIONS

Generations		N	Na	Ne	I	Ho	He	uHe	F
Gen_I	Mean	8.667	5.133	3.847	1.411	0.727	0.702	0.745	-0.045
	SE	0.126	0.435	0.352	0.097	0.051	0.034	0.036	0.059
Gen_II	Mean	23.667	4.600	3.335	1.273	0.743	0.667	0.681	-0.100
	SE	0.126	0.254	0.222	0.081	0.059	0.038	0.039	0.049
Gen_III	Mean	48.467	4.400	3.136	1.211	0.732	0.643	0.650	-0.138
	SE	0.215	0.289	0.223	0.088	0.050	0.041	0.042	0.032
Gen_IV	Mean	14.000	4.067	2.858	1.133	0.681	0.618	0.640	-0.106
	SE	0.000	0.267	0.221	0.074	0.040	0.031	0.033	0.048

TABLE 3.15 – SUMMARY STATISTIC OF GENETIC DIVERSITY BY GENERATION

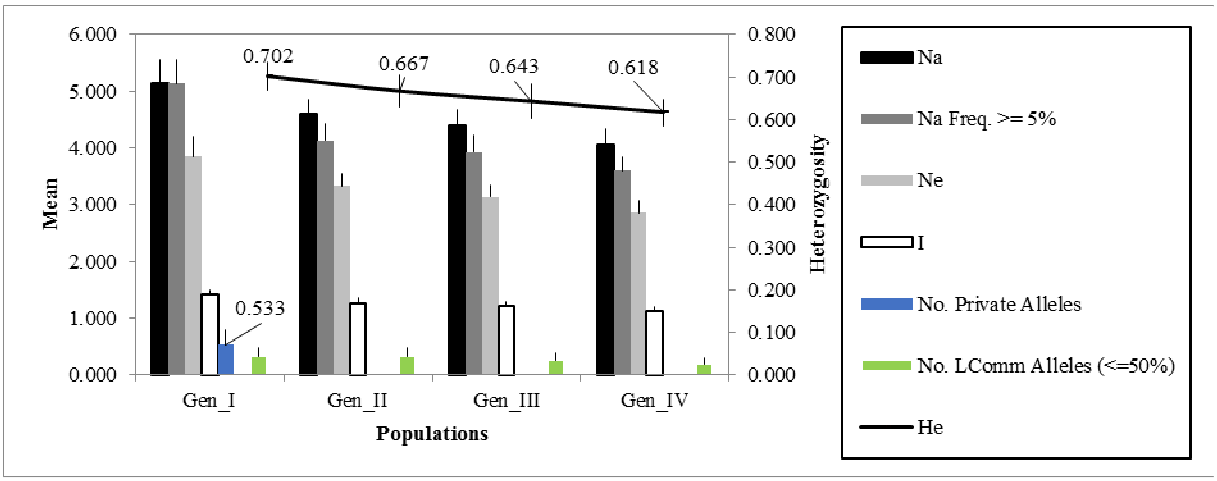


FIG 3.15 – ALLELIC PATTERNS ACROSS GENERATIONS

There were no significant deviations at single loci after Bonferroni correction (P>0.003). A summary of chi-squared test for Hardy-Weinberg equilibrium is reported in table 3.15. Number of linked loci by generations (table 3.16) rapidly increase from founders to generation III. This number strongly decrease in generation IV, but this is probably due to the low number of sampled individuals in the last generation.

Generations	Locus	ChiSq	Prob	Signif	Generations	Locus	ChiSq	Prob	Signif
Gen_I	cxx20	5.14	0.53	ns	Gen_III	cxx20	6.99	0.32	ns
	G10M	8.77	0.89	ns		G10M	11.15	0.35	ns
	G10P	16.75	0.33	ns		G10P	19.56	0.03	ns
	G10X	9.09	0.52	ns		G10X	20.37	0.03	ns
	G1D	14.25	0.51	ns		G1D	14.04	0.17	ns
	Mu11	4.78	0.91	ns		Mu11	42.74	0.00	ns
	Mu15	7.75	0.65	ns		Mu15	25.99	0.00	ns
	Mu23	12.94	0.61	ns		Mu23	23.16	0.01	ns
	Mu50	15.30	0.43	ns		Mu50	5.27	0.87	ns
	Mu59	39.75	0.31	ns		Mu59	16.64	0.08	ns
	G10C	17.24	0.07	ns		G10C	0.37	0.95	ns
	G10H	0.43	0.51	ns		G10H	0.30	0.59	ns
	G10L	2.69	0.44	ns		G10L	7.23	0.06	ns
	Mu09	12.00	0.68	ns		Mu09	20.40	0.16	ns
	Mu10	3.07	0.38	ns		Mu10	4.91	0.18	ns
Gen_II	cxx20	16.27	0.01	ns	Gen_IV	cxx20	3.06	0.80	ns
	G10M	22.65	0.01	ns		G10M	0.83	0.84	ns
	G10P	10.09	0.43	ns		G10P	7.68	0.66	ns
	G10X	18.90	0.04	ns		G10X	14.58	0.15	ns
	G1D	17.60	0.28	ns		G1D	9.98	0.44	ns
	Mu11	13.63	0.19	ns		Mu11	7.83	0.25	ns
	Mu15	17.56	0.06	ns		Mu15	16.01	0.10	ns
	Mu23	13.20	0.21	ns		Mu23	5.26	0.51	ns
	Mu50	14.09	0.17	ns		Mu50	13.16	0.59	ns
	Mu59	17.72	0.06	ns		Mu59	1.47	0.69	ns
	G10C	9.31	0.16	ns		G10C	0.56	0.90	ns
	G10H	0.34	0.95	ns		G10H	14.46	0.00	ns
	G10L	0.89	0.83	ns		G10L	3.14	0.37	ns
	Mu09	15.83	0.39	ns		Mu09	7.69	0.66	ns
	Mu10	5.12	0.16	ns		Mu10	2.94	0.40	ns

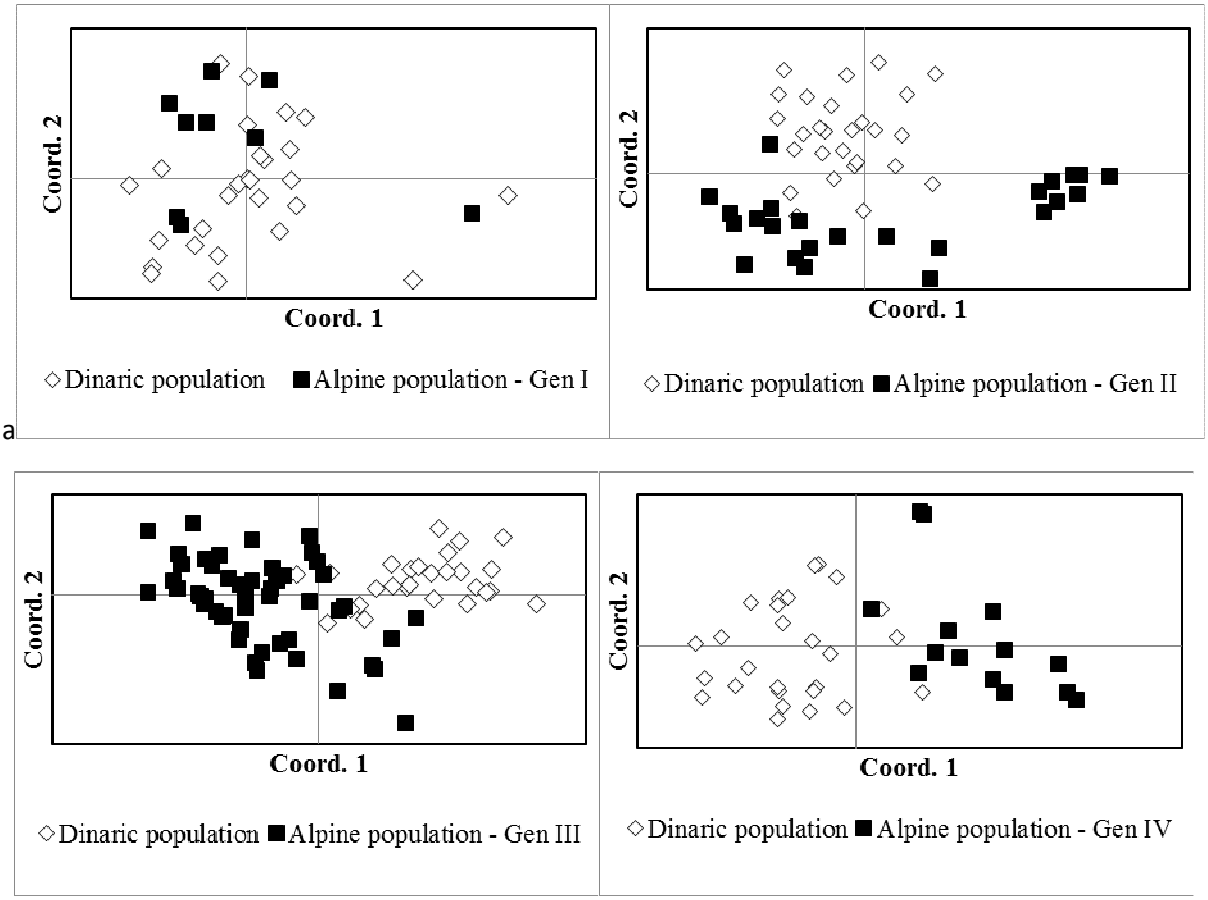
TABLE 3.15 - DEVIATIONS FROM HARDY–WEINBERG EQUILIBRIUM (HWE) BY GENERATIONS

Generations	cxx20	G10M	G10P	G10X	G1D	Mu11	Mu15	Mu23	Mu50	Mu59	G10C	G10H	G10L	Mu09	Mu10	TOT
Gen_I	1	1	1	2	0	1	1	2	0	0	2	1	3	3	4	**22**
Gen_II	9	10	12	7	10	10	10	10	3	4	13	4	10	12	8	**132**
Gen_III	10	11	10	9	12	10	9	9	9	5	11	4	6	12	7	**134**
Gen_IV	3	1	5	4	5	2	4	3	5	1	4	2	2	4	5	**50**

TABLE 3.16 - PAIRWISE LINKAGE DISEQUILIBRIUM (LD): NUMBER OF LINKED LOCI PER LOCUS BY GENERATIONS

The principal component analysis (PCA) shows a slight but clear differentiation in the allelic distribution over generations (fig 3.16) between genotypes of bears belonging to the Dinaric populations (black squared symbols) and genotypes of bears offspring of the reintroduced population (white rhomboidal symbols). In the first PC-plot only founders (Gen I) are represented, while genotypes of their descendants (Gen II, Gen III and Gen IV) are in the following graphs. We used bears dispersing from Slovenia and sampled in eastern Italy as representatives of the Dinaric population. As expected, we obtained low values of variation explained by the first three axes. This is easily explained since all bears belong to the same original Dinaric population. However, while founders genotypes do not much differ from genotypes of their population of origin, there is some kind of differentiation in the allelic distribution of their descendants, and it has become evident in only 15 years since the reintroduction.

a



Gen_I			Gen_II			Gen_III			Gen_IV		
Axis	**%**	**Cum %**	**Axis**	**%**	**Cum %**	**Axis**	**%**	**Cum %**	**Axis**	**%**	**Cum %**
1	9.58	9.58	**1**	12.95	12.95	**1**	10.03	10.03	**1**	10.79	10.79
2	7.43	17.01	**2**	8.53	21.48	**2**	6.90	16.93	**2**	7.69	18.48
3	6.61	23.61	**3**	7.09	28.57	**3**	6.31	23.24	**3**	7.26	25.75

FIG. 3.16 AND TABLE 3.17- PRINCIPAL COMPONENT ANALYSIS (PCA) BY GENERATION AND PERCENTAGE OF VARIATION EXPLAINED BY THE FIRST THREE AXIS

Measures of pairwise relatedness, reported in table 3.18, increased from the first generation (Gen I) to the third one (Gen III) and decreased in the last generation (Gen IV). As for the principal component analysis, this is likely a bias due to the lowest number of bears in the last generation, which is likely not representative of the real situation. These results indicate that the expected proportion of shared alleles between pairs of individuals that are identical by descent increase over generations.

	Gen_I			Gen_II			Gen_III			Gen_IV		
	RI	**LRM**	**QGM**	**RI**	**LRM**	**QGM**	**RI**	**LRM**	**QGM**	**RI**	**LRM**	**QGM**
Mean	**-0.063**	**-0.129**	**-0.129**	**-0.020**	**-0.044**	**-0.044**	**-0.010**	**-0.021**	**-0.021**	**-0.036**	**-0.077**	**-0.077**
Median	-0.062	-0.132	-0.118	-0.034	-0.085	-0.060	-0.020	-0.054	-0.034	-0.047	-0.124	-0.112
SD	0.043	0.084	0.135	0.112	0.260	0.325	0.089	0.201	0.241	0.100	0.241	0.296
SE	0.007	0.014	0.023	0.007	0.016	0.020	0.003	0.006	0.007	0.011	0.025	0.031
Min	-0.179	-0.287	-0.429	-0.223	-0.472	-0.646	-0.221	-0.496	-0.675	-0.194	-0.417	-0.545
Max	0.025	0.054	0.126	0.282	0.634	0.736	0.563	0.734	0.756	0.399	0.799	0.831

TABLE 3.18 – COEFFICIENT OF PAIRWISE RELATEDNESS (RXY) SUMMARY USING THREE ESTIMATORS (RI= RITLAND 1996 ; LRM= LRM=LYNCH & RITLAND 1999; QGM= QGM=QUELLER AND GOODNIGHT 1989)

Demographic estimates of contemporary effective population size (Ne) were extremely low and did not vary considerably using the two different methods. Genetic estimates of Ne ranged from 5.6 (CIs 3.9/3.4-7.2/9.0, 95% CIs) using the linkage disequilibrium method, to 8.1 (CIs 6.5/4.8-4.8/11.7, 95% CIs)(table 3.19).

LOWEST ALLELE FREQUENCY USED	**0,050**	**0,020**	**0,010**	**0+**
LINKAGE DISEQUILIBRIUM METHOD				
Harmonic Mean Sample Size	45	45	45	45
Independent Comparisons	1207	1304	1304	1304
OverAll r^2	0.061	0.056	0.054	0.054
Expected r^2 Sample	0.023	0.023	0.023	0.023
Estimated Ne^	**5.6**	**7.3**	**8.1**	**8.1**
95% CIs for Ne^ - Parametric	3.9	5.7	6.5	6.5
	7.2	8.9	9.9	9.9
95% CIs for Ne^ - JackKnife on Loci	3.4	4	4.8	4.8
	9	10.6	11.7	11.7
MOLECULAR COANCESTRY METHOD				
Harmonic Mean Sample Size				45
OverAll f1^				0.088
Estimated Neb^				**5.6**
95% CIs for Ne^ - JackKnife on Loci				3.4
				8.5

TABLE 3.19 - ESTIMATION OF CONTEMPORARY EFFECTIVE POPULATION SIZE (NE) USING FOUR DIFFERENT METHODS

The simulation of effective population sizes (Ne) in 100 and 500 years are reported below. Neff provided Ne of 24.31 in 100 years and 26.31 in 500 years. The loss of heterozygosity is relatively high per year (-0.0019 in 100 years and -0.0026 in 500 years) and generation (-0.0206 in 100 years and -0.019 in 500 years) (table 3.20 and fig. 3.17).

PARAMETERS	**100 YEARS**			**500 YEARS**		
	VALUES	**95% lower CI**	**95% upper CI**	**VALUES**	**95% lower CI**	**95% upper CI**
Slope of heterozygosity loss per year	**-0.0019**	-0.0036	-2x10-	**-0.0017**	-0.0026	-8x10-
Slope of heterozygosity loss per generation	**-0.0206**	-0.0374	-0.0037	**-0.019**	-0.0288	-0.0092
mean generation length	**11.2**	10.6	11.9	**10.8**	10.5	11.4
Ny[simulation]	**125.52**	263.87	250.42	**290**	278.08	301.93
Ny[calc]	**225.87**	-	-	**225.87**	-	-
Ne[simulation]	**24.31**	20.51	28.12	**26.31**	22.71	29.9
Ne[calc]	**20.01**	-	-	**20.81**	-	-

TABLE 3.20 - COMPARISON BETWEEN TWO POPULATIONS FOR 100 YEARS AND 500 YEARS. SLOPES OF HETEROZYGOSTY AND MEAN GENERATION LENGTHS PER YEARS AND GENERATIONS ARE CALCULATED. NY REFERS TO ANNUAL POPULATION SIZES AND NE TO EFFECTIVE POPULATION SIZES.

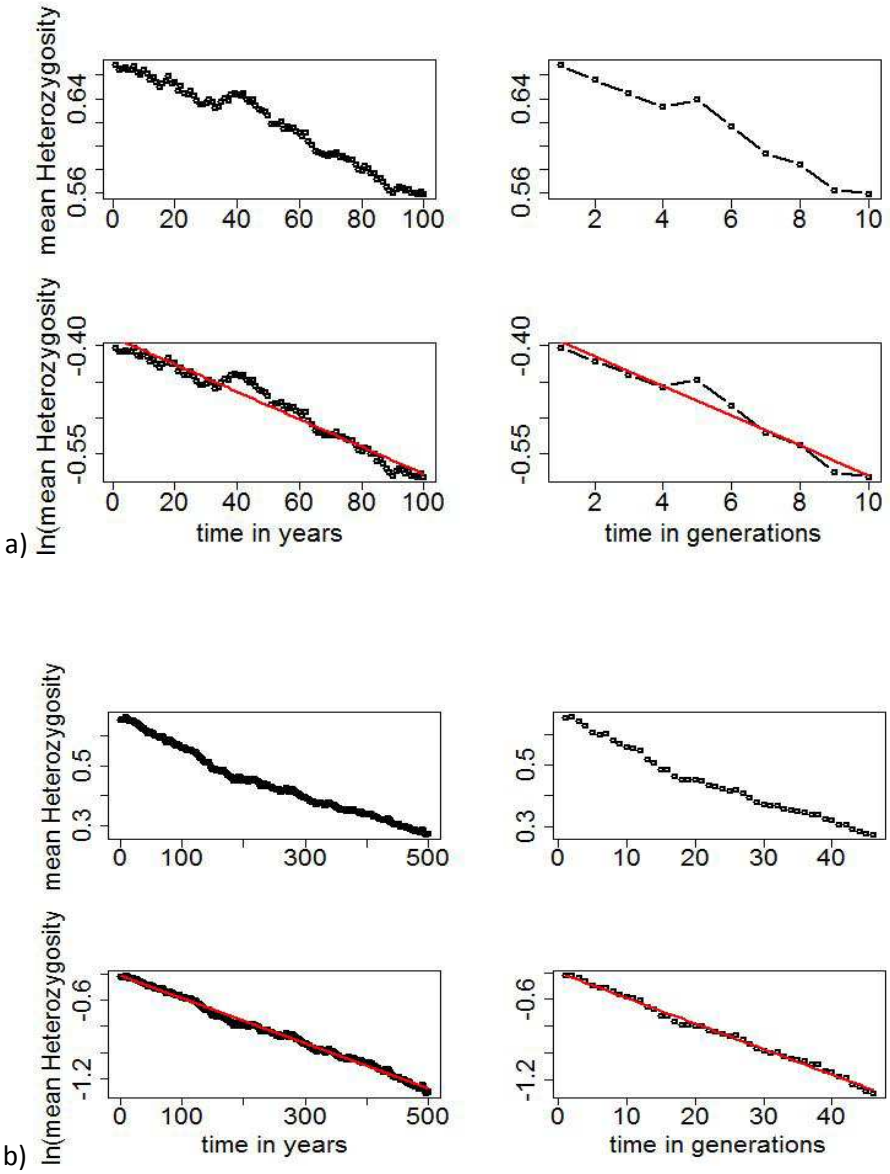


FIG. 3.17 - SLOPE OF HETEROZYGOSITY LOSS IN YEARS (LEFT) AND GENERATIONS (RIGHT). COMPARISON BETWEEN TWO POPULATIONS SIMULATED FOR 100 (A) AND 500 YEARS (B)

DISCUSSION

Various monitoring methods have been developed for large carnivores including telemetry, capture-mark-recapture, harvest data, and sign survey (Wilson & Delhay 2001, Barea-Azcò et al 2007), but non-invasive genetic sampling has recently become the preferred method for studying elusive large carnivore populations (Caniglia et al 2004, Rudnick et al 2005, Borthakur et al 2011, Tsaparis et al 2014), since it is more feasible and cost-effective (De Barba et al 2010). It also has been successfully applied to monitor populations after reintroductions (Ausband et al 2010, Stenglein et al 2010, Koelewijn et al 2010). According to the IUCN Reintroduction guidelines (1998), the ultimate goal of reintroduction projects should be the "the establishment of a self-sustaining population that requires minimal long-term management". The importance of a monitoring project, focused in identifying possible causes of success or failures, has to be performed to achieve this goal (Nichols & Williams 2006, Seddon et al 2007, Armostrong & Seddon 2008), and resulting information should be used to improve future project actions (Koelewijn et al 2010).
This study provides a good example of how genetic monitoring allows collecting demographic and population genetic data for a comprehensive knowledge of small and reintroduced populations such as brown bears in the Italian Alps. Data should include vital statistics, recruitment, mortality, geographic patterns and population genetic parameters such as genetic diversity, inbreeding, and effective populazion size.

Non-invasive genetic sampling and genotyping suitability

Non invasive genetic sampling (NGS) and microsatellite markers have been proved a powerful tool for studying wildlife population of elusive and endangered species (Taberlet et al 1999, Waits & Peatkau 2005, Bellmain et al 2005, Luikart et al 2010) and it has been widely employed in bear research and management (e.g Woods et al 1999, Mowat & Strobeck 2000, Poole et al 2001, Kendall et al 2008, Robinson et al 2007, Karamanlidis et al 2007, Bellmain et al 2005, 2007). However, the feasibility of this approach depends on project goal and study population (Piggott & Taylor 2003). A pilot study was conducted to evaluate the potential of NGS for monitoring the small reintroduced Alpine population (De Barba et al 2010) using 10 microsatellite markers. Results demonstrated that 10 microsatellite loci were sufficient to identify individuals and parent-offspring relationships, that the NGS approach was feasible, and that the integration of different sampling methods increased the probability of detecting the majority of individuals (De Barba et al 2010). However, populations are not stable and factors determining the efficiency of one approach can vary over time together with changes in population dynamics. Consequently, it is recommended to adjust approaches according to population dynamics over time.
Because of loss of released individuals, the occurrence of offspring and the increasing relatedness among individuals in the reintroduced population, we increased the number of microsatellite markers from 10 to 15, in order to enhance the power in identifying parent-offspring relationships. We compared the discriminatory power in individual identification calculated through PID and PIDsibs values and the power in excluding parents from the dataset through P1 and P2, when using 10 and 15 loci. PID and PIDsibs using 10 loci were $4.0x10^{-10}$ and $1.4x10^{-4}$, while were $4.4x10^{-13}$ and $6.9x10^{-16}$ using 15 loci. The probability of parent exclusion with both parent known did not significantly change using 5 more loci (~0.999 using 10 and 15 STRs), while increased with only one parent known (from 0.979 using 10 loci to 0.992 using 15 loci). Thus, even if 10 loci were proved to be sufficient to reliably distinguish individuals in 2008 (De Barba et al 2010), the addition of five more microsatellite loci provided higher discriminatory and exclusion power, both needed after the recent increase in the number of close relatives in the population. However, we found a few reference genotypes in the central Alps with all but 2 or 3 loci in common. Moreover, a few parentage assignments were discarded as considered unreliable on the basis of incongruent assignments using two different software. We believe all multilocus genotypes to be accurate since single genotypes have been identified multiple times from multiple samples, all samples have been independently analyzed, and further 4 amplifications at mismatching loci have been performed to confirm alleles.
Given these results, we stress the importance of further increasing the number of markers and consequently the information content, despite the high discriminatory and exclusion power using 15 microsatellite loci. Single nucleotide polymorphisms (SNPs) would be a good choice, since they are expanding their popularity in wildlife monitoring projects (Vignal et al 2002, Brumfield et al 2003) and proved to be efficient in individual identification (Seddon et al 2005, Kraus et al 2015, Fitak et al 2016) and parentage assignments (Tokarska et al 2009, Hauser et al 2011, Fernandèz et al 2013,Wright et al 2015, Kaiser et al 2017).
The use of a non-invasive sampling approach proved to be feasible at the beginning of the project (data from 2002 to 2008) and allowed sampling a number of genotypes that provided a minimum count of bears that was within the range

of field-based observation estimates (De Barba et al 2010). One of our goals was to determine whether or not the sampling protocol was still efficient in determining population size, despite the numerical and geographical expansion and the use of different sampling procedures in different areas, calibrated according to the local context. Intense sampling has been applied only in areas of stable presence, and the risk was to miss the sampling of individuals dispersing outside the core area. Instead, the integration of a regular and continuous opportunistic sampling in the expanding area was implemented. Annually, only a few bears escaped the genetic sampling, but they were sampled in the following years, allowing to infer more accurate annual minimum counts. However, simulations with CAPWIRE program, indicated that the expected number of observed genotypes in 2010, and between 2013 and 2016, was higher than the observed one. This suggests that the number of bears was underestimated, especially in the last 4 years, when bears were dispersed throughout the Alps. This is likely due to the greater difficulty in sampling all dispersing bears using the opportunistic sampling approach alone.
We noticed a high differentiation in genotyping success among different DNA sources. Despite 90% alcohol was demonstrated to be the most efficient method to preserve fecal DNA (Murphy et al 2002), scats showed the lower genotyping success (28.23%). Scats are an important source of DNA (2292 samples collected), especially for identifying cubs, which often escape the sampling through hair-traps. Therefore, we emphasize the importance of finding a more efficient protocol for the genotyping of fecal DNA. We are aware that a percentage of analyzed samples may not belong to the target species, since a genetic pre-screening of samples to confirm the species (e.g. amplifying the COI species-specific mitochondrial sequence) was performed only for a proportion of samples, in order to reduce time and costs of the analysis However, bear scats are easy to distinguish and collectors have gained experience in scat identification since the sampling started. Thus, we are confident that non-target samples are likely to be a low percentage.

Reproductive success and mating system

Using DNA as a tag to identify individuals, we were able to build a detailed life table and a pedigree of the brown bear population in central Italian Alps. The ability to derive wild pedigree is one of the most important benefits of genetic monitoring for small and reintroduced populations, since allows not only to track demographic and genetic changes, while also providing insight into the mating system and social behaviour of the population. Many informations, such as age of first reproduction for both sexes, the number of litters of single individuals, and variance in reproductive success among founders, could not have been gained using traditional field-methods alone. 48 litters were detected for a total of 93 cubs, thus we deduced that the majority of founders successfully reproduced after being released (2 out of 3 males and 5 out of 7 females). However, 3 females alone (Daniza, KJ1, and KJ2) gave birth to the 40.8% of all cubs (38 out 0f 93) and 3 males (Gasper, Joze and MJ5) were fathers of 75.2% of offspring, especially Gasper, that alone gave birth to the 44% of the population. At the beginning of the project only the older male founder reproduced (Joze), and the second male, Gasper, had its first litter at 7 years (De Barba et al 2010). Similarly, Gasper continued siring all cubs, and only when it was old, other males of the second generation (e.g. MJ5) started reproducing. The involvement of native-born individuals in reproduction is one of the criteria for the short-term success of reintroduction efforts (IUCN 1998, Morell 2008, Seddon et al 2007). Thus, even if almost all founders contributed to reproduction, the reproductive success was skewed towards a few individuals, especially for males. These results suggest a hierarchy of dominance, which is expected to increase relatedness and the number of inbreeding events in the reintroduced population, as described by values of coefficients of pairwise relatedness (Rxy) over generations and the pedigree reconstruction. Males competing for mating and older and larger individuals having higher reproductive success is a wel- known behavior in many promiscuous mammals, including bears (Zedrosser et al 2007). Therefore, our results underline the importance of considering the mating system in translocations, selecting founders of similar age and size to avoid distortions in individual reproductive success (De Barba et al 2010), and suggest to take further translocations into consideration in a long-term perspective.
First year of reproduction for females was at the age of 3. This is considered an early stage of female sexual maturity for bears, since it was not observed in North American populations (Craighead et al 1995). However, the year of transition to the reproductive age was the same in central Austria (Zedrosser et al 2004). Also, the inter-birth interval of 2 years is consistent with other European populations (Dahle & Swenson 2003) but shorter than in North America (Schwartz et al 2003). Mean litter size, that ranged between 1.5 and 2.3, was comparable to other bear populations (Frkovic et al 2001, Swenson et al 2003). One multiple paternity was documented (cubs MJ2J1 and MJ2G1), and similar cases have been previously documented in brown bears (Bellmain et al 2006, Craighead et al 1995).

Demographic and geographic expansion

High survival and reproductive outputs are essential for the establishment of a self-sustaining population and hence the success of a reintroduction (Griffith et al 1989, Ostermann et al 2001). Over the 16 years after reintroduction, the bear population in central Alps approximately quintupled in size thanks to the high number of reproductive events and relatively high survival rates, that ranged from 0.79 for juveniles to 0.89 for adults. A remarkable growth rate was observed (current level of approximately 20%). Although the population growth was positive, mortality was high and half of the recorded deaths turned out to be human-induced, due to car collisions, poaching, or poisoning. All of these causes have been described as major threats to bear survival in other studies in human-dominated landscapes (Swenson et al 2000). It is remarkable that a number of deaths are caused by legal culling or negative consequences of capture attempts, in Italy and neighboring countries. These decisions have been taken in order to safeguard public opinion since acceptance of bear occurrence is not widespread and the opposition recently got worst after two cases of injury to humans (Tosi et al 2016). Alternatively, problematic bears have been moved into captivity, an action that, from an ecological prospective, it is not different from culling, because the possible reproduction is prevented.

Not only the demographic trend but also patterns of geographic expansion will affect the long-term self-sustainability of a reintroduced population and therefore should be investigated. For example, inbreeding or resource and mate competition are tipically avoided through natal dispersal (Wolff 1993), which therefore has an important role for gene flow and post-release mortality (Rhodes & Latch, 2013). In our study, spatial inferences can be potentially biased by unequal sampling intensity, since different sampling approaches were applied depending on local context and stable or unstable bear presence. However, a high proportion of the population was sampled in each year, allowing us to make general conclusions about temporal-spatial distribution and movement patterns of single bears.

Females were always detected within a limited distance from the translocation area during the whole study period. Females have been observed to disperse in other expanding bear populations (Jerina & Adamic 2008, Swenson et al 1998), with important implications for colonization patterns and genetic connectivity, but this is not the case for the Alpine population. A reason for range fidelity of females could be the highly human-dominated area, the Adige valley with its highway, which seems to be a barrier preventing female dispersal. Bears can cross human-dominated habitat patches, but human activity interferes and limit dispersal (Proctor et al 2004). The presence of the Adige valley could have fewer effects on dispersal capacity of males: male bears, offspring of founders, have certainly crossed this barrier since were detected in the neighboring administrative regions starting from 2007, seven years after the reintroduction. The presence of Alpine bears in eastern Italy demonstrates that, despite high level of habitat fragmentations, bears are able to travel long distances and move towards the Alps crossing the Adige valley, and prove the existence of ecological corridors in the Italian Alps. Our results are consistent with other bear studies, which documented high philopatry of females and greater dispersal capacity among males (Dahle & Swenson 2003, Proctor et al 2004, Schwartz et al 2003, Støen et al 2006). The dominant behavior of adult males in the core area has probably caused the exodus of juvenile males, which is a scheme often observed in many carnivores species (Kruuk & Moorehouse 1991, Creel & MacDonald 1995). Data on bear deaths, acquired in this study, are insufficient to scientifically verify which are major threats to dispersing bears. However, the roaming in newly acquired territory usually enhance the probability of dispersing animals to be killed in a road traffic accident (Koelewijn et al 2010).

On the other side of the Alps, a number of bears dispersing from the Dinarics have been sampled since 2011. As for the Alpine population, dispersal was male-biased. The number of bears sampled in eastern Italy (in Friuli-Venezia Giulia and Veneto) was variable each year, but the overall positive trend is promising for a stronger colonization of this area in the future. The high turnover of bears each year may be caused by the absence of females in the area, which prevents a stable colonization. It is remarkable that only 1 bear of Dinaric origin (M5) was sampled beyond the Adige valley. Observing the geographical distribution of Alpine bears, ecological corridors seems to exist. Therefore, the absence of Dinaric bears west of the Adige valley is likely due to the long distance to reach females in the Alps. Thus, Dinaric bears probably tend to move back to their territory of origin.

We tested the occurrence of geneflow between the reintroduced Alpine population and the population of origin in the Balkans using Dinaric dispersing male bears, sampled in eastern Italy, as putative fathers. Our hypothesis was that, given the recent high dispersal of males, matings between the two disjoint nuclei could have happened following the recent expansion of both populations. However, no bear born in the central Alps had parents of Dinaric origin, thus, despite the recent spatial overlap of the two populations, the genetic connectivity was not re-established.

Genetic diversity, inbreeding and effective population size

A positive demographic trend is not the only factor that takes part in the success of a reintroduction. Determining the rate of loss in genetic diversity and changes in genetic composition is of great importance since genetic diversity has substantial effects on the evolutionary potential and viability of a population (Waits 1999). Populations with high genetic diversity have good evolutionary potential and thereby great prospects to survive in the long-term (Reed & Frankham 2003). In contrast, populations with low genetic diversity might not be able to adapt to environmental changing factors (e.g. climate change, the spread of a disease…), since alleles have been lost through genetic drift (Slatkin 1987). Thus, measuring parameters of genetic diversity and composition and identifying factors that cause changes are needed to apply proper conservation strategies for alleviating further losses of genetic variation (Rhodes & Latch 2013).

The significant decline in both expected heterozygosity (He from 0.702 to 0.618) and allelic richness (Na from 5.133 to 4.067), indicate that the reintroduced brown bear population has lost genetic diversity due to drift in less than four generations, as also visually described by the PCA analysis. Since we are certain that the brown bear population in the Italian Alps is reproductively isolated, the linkage disequilibrium (LD) at neutral loci may grow as a result of genetic drift (Nei and Tajima, 1981): as expected, the number of linked loci increase over generations.

However, the level of genetic diversity still remains relatively high if compared with other isolated and endangered bear populations that have suffered severe historical demographic bottlenecks, such as the Apennine population (He 0.44, Na 2.44) and the Cantabrian population (He 0.28, Na=1.75), but its low if compared with the population of origin in Slovenia (He 0.73, Na 6.68) (Skrbinsek et al 2012), due to the founder effect. The loss of genetic variation due to the founder effect and genetic drift has been documented in a variety of species (Ewing et al 2008, Mock et al 2004, Wisely et al 2008.

In small isolated populations the rate of inbreeding can rise rapidly, depending on the effective population size (Ne)(Frankham et al 2002, Waples 2002). In real populations, Ne is determined by critical life history and demographic parameters (e.g. historical fluctuations in population size, variance in reproductive success and sex ratio; Frankham, 1995). In the Alpine area, the population is growing and the sex ratio is equally divided, but reproductive success is strongly skewed towards a few founder individuals. As a consequence, there is a progressive increase in relatedness among individuals. Under persistent isolation, inbreeding could be the most important risk factor for survival (Keller & Waller 2002): data from other bear and wolf populations (Laikre 1999, Liberg et al 2005) indicate that inbreeding can have strong negative effects. Several cases of inbreeding are already evident in the pedigree reconstruction. Since 2014, when all founders have died, the risk of inbreeding is even higher, because now all bears are closely related.

Ne of the translocated population has remained extremely small, as recorded in a previous study of this population (De Barba et al 2010), and its fare below the minimum of 50 recommended for short-term conservation (Allendorf & Riman 2002). Therefore, counteracting losses in genetic variation and negative effects of inbreeding will depend on the ability to upgrade the effective population size and restore the geneflow with the Dinaric population. Under this condition of isolation, expected heterozygosity will decrease exponentially to 0.50 in only 100 years and will reach alarming values in 500 years, as described by the simulation performed in this study.

CONCLUSIONS

A recent study (Tosi et al. 2016) talked about the reintroduction of bears in the Italian Alps using enthusiastic terms, since the population numerically increased and expanded its geographic area. Our data are in agreement with the previous study: from a demographic point of view, this reintroduction can undoubtedly be considered a great success. So far, few bear reintroduction efforts have occurred, and fewer have been successful since presents many challenges (low population growth, low genetic variability, strong homing instinct and increased mortality after translocation)(Clark et al 2002). However, nobody provided updated informations on the genetic status of this population or investigated the possible reconnection with the Dinaric population of origin. Loss of genetic variability, increase of relatedness and inbreeding are important threats to the survival of this population in the long-term. Therefore, intense efforts are needed to re-establish the ecological connection in the Alps, in order to allow bears to move in the territory without running into ecological barriers, such as highways (Coffin 2007, van der Ree, 2011). Many studies have been performed to identify best locations for bridge underpasses (van der Ree et al 2007, Clevenger & Waltho 2004, Lesbarrères & Fahrig 2012, Bond & Jones 2008), that could be integrated into the two major highways crossing the area in order to reduce collisions with cars. To facilitate the geneflow between the Alpine and Dinaric populations, a further translocation of a couple of females in Veneto or Friuli-Venezia Giulia should be considered, counteracting the homing instinct of Dinaric bears. Moreover, death of anthropic origin are substantial and should be reduced. Finally, a close cooperation among all different administrative bodies involved in the protection of bear in the Alps, in Italy and abroad, is required and a complete genetic databank should be shared. Since several conservation genetic laboratories are involved in the analysis of samples, same microsatellite markers, with a calibration of alleles, should be used in order to compare genotypes. Alternatively, microsatellite could be replaced by markers which do not require allele calibration, such as SNPs.

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CHAPTER IV - Testing a new SNP-chip on the Alpine and Apennine brown bear (*Ursus arctos*) populations using non-invasive samples

ABSTRACT

Brown bears in Italy persist in two isolated populations, one in the Alpine and the other in the Apennine mountain range. Both are threatened and elusive. Non-invasive genetics provides a good way to monitor the populations. Microsatellites (STRs) have been the marker of choice for non-invasive genetic monitoring, but due to non-invasive bad quality samples, these analyses were plagued by low amplification rates and genotyping errors. Moreover, to compare microsatellite genotypes, allele calibration is needed between laboratories, leading to difficulties in individual identification. In contrast, SNP genotyping is directly comparable between laboratories, and more sensitive and accurate. Here we test a 96-marker SNP chip developed for the Scandinavian brown bear population on the Italian populations. A subset of these SNPs was found informative and could reliable confirm species, sex and, only in the Alpine population, distinguish individuals. A total of 51 informative SNPs provided better resolution power than 15 STRs, used in the routine monitoring of the Alpine population in Italy. In contrast, only 15 SNPs were found to be informative for the Apennine population, which did not have enough resolution to discriminate individuals and were less informative than 11 STRs. While highly useful in the Alpine population, additional SNP markers must be included to reach the same level of resolution in the Apennine population.

INTRODUCTION

Elusive and rare species are difficult to monitor, therefore non-invasive genetics are commonly employed in carnivore management (Gervasi et al 2008; Kindberg et al 2011; Tsaparis et al 2014). Non-invasive genetic sampling enables the populations to be studied without capture or other disturbances (Taberlet et al1999). By establishing individual genotypes it is possible to quantify the minimum number of individuals, and estimate the effective population size, demographic parameters and genetic structure (De Barba et al 2010; Karamanlidis et al 2012). Moreover, identifying individuals is the first step for kinship reconstruction and estimates of reproductive success and inbreeding prevalence (Haanes et al 2013; Brzeski et al 2014).

However, non-invasively collected material is often degraded, holding low-quality DNA (Piggott and Taylor 2003). As a consequence, population genetic studies based on non-invasive sampling are fraught with many difficulties. In the last two decades, microsatellite (STRs) have been the marker of choice for monitoring genetic studies with non-invasive samples (Taberlet and Luikart 1999; Broquet et al 2007). But STR analyses typically show low amplification rates and high levels of genotyping error (Dewoody et al 2006). Frequent genotyping errors, like allelic drop-outs (ADO) and false alleles (FA), can result in identification of erroneous genotypes with a consequent overestimation or underestimation of individuals (Creel et al 2003).

SNPs (Single Nucleotide Polymorphisms) have a number of features that make them superior to STRs. This has led to SNPs being preferred by many carnivore monitoring projects (Seddon et al 2005; Kraus et al 2015; Fitak et al 2016). Firstly, SNPs markers require shorter DNA fragments and are thus less sensitive to degradation (Morin et al 2004; Seddon et al 2005). Second, SNPs are less prone to genotyping errors (Anderson and Garza 2005). Third, the simple mutational dynamics in SNPs highly reduce the risk of homoplasy (Vignal et al 2002) when compared with STRs. Fourth, SNPs are based on single nucleotide changes and thus, unlike STRs, do not require calibration of allele calling across different laboratories (Vignal et al 2002). Finally, the advent of next-generation sequencing technologies has made SNP markers more accessible, even in terms of costs (Kumar et al 2012).

Despite the advantages, a SNP is typically bi-allelic and less informative than a multiallelic STR. But as more SNPs can be analyzed, the resolution of a SNP panel typically widely exceeds that of an STR panel (Seddon et al 2005; Hauser et al 2011). Moreover, SNPs have lower mutation rates than STRs and ascertainment bias can arise when transferring markers across populations.

Recently, Norman et al (2013) developed a panel of 96 high quality SNPs comprising 85 autosomal SNPs, 7 sex chromosome markers, and 4 mtDNA markers. Here, we identify the subset of SNPs informative for Italian populations and test its resolution and efficiency of species confirmation, sex determination and individual identification. Moreover, we compare the power of individual identification of SNPs with that derived from the 15 and 11 STRs currently used in the Alpine and Apennine population, respectively. Finally, we compare the resolution power of SNPs between Scandinavian population, for which the markers were developed, and Italian populations, in order to highlight differences caused by the ascertainment bias that is expected when transferring SNPs across populations.

MATERIAL AND METHODS

Sample collection, storage and selection

Forty-five samples (23 from the Alpine population and 22 from the Apennine population), belonging to 45 individuals, identified from STR analyses of hair samples, were selected. All individuals were sexed using sex-specific DNA fragments: Alpine samples were sexed using the Amelogenin locus (AMG) (Ennis and Gallagher 1994) and the SRY locus (Fechner 1996), while Apennine genotypes used only the AMG. To improve the quality of the SNP genotyping, we selected samples with the highest percentage of positive PCR amplifications (mean=96%, SE=0.010). STR genotyping errors in the consensus genotypes were reduced by a multi-tube approach (Taberlet et al 1996) that included four PCR replicates from the same extract. Genotyping errors were calculated on replicates with GIMLET v.1.3.3 (Valière 2002). When genotyping errors were found, 4 more PCR replicates were added. A reliability score R for each multilocus genotype was calculated with RELIOTYPE (Miller et al 2002). We selected only genotypes with R>0.95.

STR genotyping

The genetic monitoring program of the Alpine bear population started in 2002 following the translocation of 9 bears belonging to the Dinaric population. Until 2014 hundreds of hair and faecal samples have been non-invasively collected with different sampling method: opportunistically during field patrolling, by the use of baited-hairtraps, on rub-trees and during inspections of damages to beehives and livestocks, with the aim of sampling all present bears including new born individuals. Samples were delivered to the Institute for Environmental Protection and Research (ISPRA), Italy, where they were immediately stored at -20C°.
Ten STR loci were used for individual identification and sex was determined using the Amelogenin locus (AMG) (Ennis & Gallager 1994). We implemented the protocol adding 5 STR loci to perform parentage analysis and the Sex-determining Region Y (SRY) (Fechner 1996) to confirm sex. The 15 STR loci were selected with the best compromise between highest individual discrimination power (lowest probability of identity among siblings: PIDsibs, Waits, Luikart & Taberlet 2001) and highest number of loci in common with other abroad laboratories that investigates brown bears in the Alps, allowing exchange of data on cross-border bears. Extractions were conducted using the Zymo ZR-96 Quick g-DNATM protocol using manufacturer's instructions. List of primers, amplification and sequencing protocols for individual identification and sex determination are described in details in De Barba et al 2010b, while the implementation methods are expounded in Davoli et al 2018. All DNA samples were eluited in 200 µL of eluition buffer and stored at -20°. The presence of matching genotypes within the dataset was checked with the command *Matches* in the option *Multilocus* from GeneAlEx 6.4 software (Peakall and Smouse 2012) menu. A genotype-databank was developed, consisting of all bears sampled in the study area between 2002 and 2014

DNA extraction and SNP genotyping

DNA of the 45 selected samples was re-extracted at ISPRA, using the QIAGEN DNeasy® Blood & Tissue Kit (Qiagen inc, Hilden, Germany) according to manufacturer's instructions. Extractions were performed in dedicated rooms, after sterilizing the work material under UV-light hoods. A negative control was included.
The re-extracted samples were sent to the Swedish University of Agricultural Sciences (SLU), Sweden, where SNP genotyping was carried out on a BioMark (Fluidigm Corporation, San Francisco, USA) following manufacture's instruction with the exception of number of pre-amplification cycles - 35 cycles were used instead of 14 in the polymerase chain reaction in order to accommodate for low concentrations of DNA. Positive and negative controls were included and 23 genotypes were triplicated for the estimation of genotyping errors. The analysis was performed using the 96x96 SNP panel described in Norman et al (2013) and modified in Norman and Spong (2015).

SNP and sample validation

In order to obtain a subset of reliable SNPs, we removed all SNPs and samples which produced unusable or ambiguous results. We filtered SNPs and samples following further procedures: a) we removed SNPs which gave no amplification signal in any sample (Table 4.1, step 1) and those which were monomorphic in both populations (Table 4.1, step 2), excluding Y-chromosome and mitochondrial SNPs, which are always haplotypic; b) we visually examined the genotyping clusters in a combined analysis of all 45 samples, using the Fluidigm SNP genotyping

analysis software v3.1.2, and removed all loci which showed unclear cluster affiliation or unusual clustering patterns (e.g. Fig 4.1), preventing possible errors in genotypes (Table 4.1, step 3); c) we created two different SNP subsets separating the Alpine and the Apennine samples and we removed the SNPs which appeared monomorphic in their respective populations (Table 4.1, step 4); d) we calculated the call rates and removed all samples showing ≥50% of missing data in order to remove low quality DNA samples (Table 4.1, step 5); e) we created a consensus genotype for all samples with replicates. All allele differences (e.g. replicate 1: A/T, replicate 2: A/A or replicate 1: A/A, replicate 2: T/T) among replicates were recorded as heterozygotes; f) finally, we selected only SNPs with ≥70% of call rates on consensus genotypes (Table 4.1, step 6). Only samples and SNPs which passed the screening were processed for further analyses.

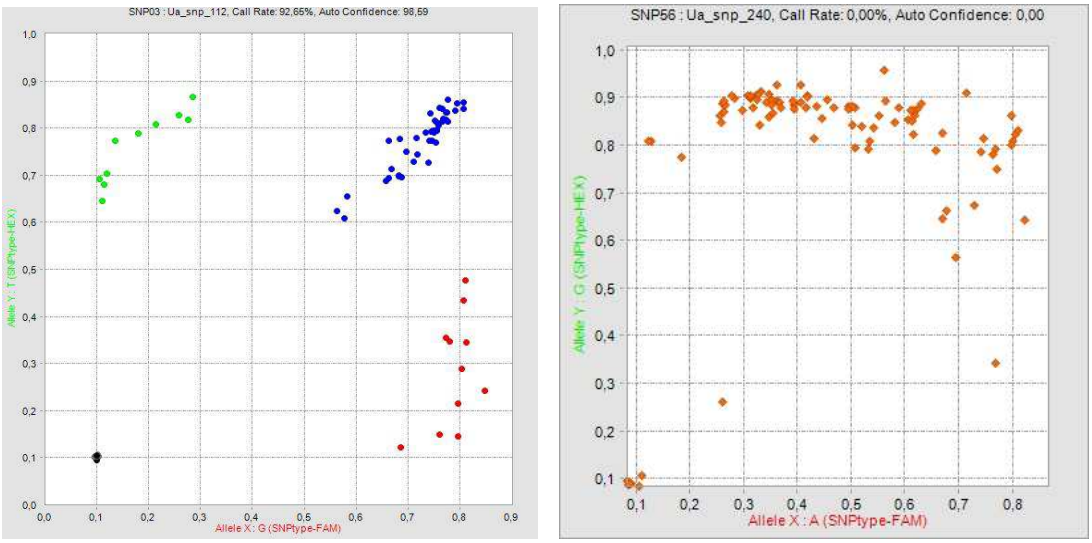


FIG. 4.1 – CLUSTER PLOTS OF A SELECTED (LEFT) AND A REJECTED (RIGHT) SINGLE NUCLEOTIDE POLYMORPHISM ON THE FLUDIGM SNP GENOTYPYING

Species confirmation and sex determination

The SNP-chip comprised four mtDNA haplotype SNPs. Mitochondrial DNA is more abundant than nuclear DNA and thus can be easily detected in degraded samples. For these reasons, SNPs on mitochondrial DNA were used for confirmation of the target species. We visually examined the genotyping clusters using the Fluidigm SNP genotyping analysis software v3.1.2. When the sample appeared in the cluster for at least 3 out of 4 mitochondrial markers, it was recorded as belonging to the target species, otherwise it was stated as unknown species. This analysis is very useful in bear monitoring, since it permits to determine if an area is frequented by the species, although it is not possible to trace the individual genotype due to DNA degradation.

Four markers on the Y-chromosome were included for sex determination. Since Y-chromosomes typically show low levels of nucleotide diversity in mammals (Hellborg and Ellegren 2004), the markers on Y-chromosome are monomorphic in our populations and are thus used for sex identification. The Fluidigm SNP genotyping analysis software v3.1.2 was used to identify clusters. Homozygous clusters are present only for male samples, whereas female samples are expected not to show any sign of amplification. When the sample appeared in the cluster for at least 3 out of 4 Y-chromosome markers, it was recorded as a male. Conversely, the absence of amplification for all 4 markers indicated that the sample belonged to a female individual. When a sample appeared in the cluster for less than 3 Y-chromosome markers, it was stated undetermined. In addition, three X-chromosome SNPs were used to validate the Y-chromosome determination of sex of males by ensuring they had no heterozygote genotypes.

Genetic variability of SNP loci and individual identification

The 85 autosomal SNPs were used for individual identification through multilocus genotyping, after removing those deemed unreliable as described in the "SNP and sample validation" paragraph.
GeneAlEx 6.4 software (Peakall and Smouse 2012) was used to perform genetic variability analysis of SNPs and STRs. Number of alleles (Na), effective number of alleles (Ne), Shannon's information index (I), observed (Ho) and expected (He) heterozygosity, unbiased expected heterozygosity (UHe) and fixation index (F) were calculated separately for the Alpine and the Apennine population. Deviations from Hardy-Weinberg equilibrium (HWE) were tested and significance levels were adjusted for multiple comparisons using a Bonferroni correction. The minor allele frequency (MAF) for SNPs was calculated manually.
We further estimated the ability of SNPs to distinguish individuals using the probability of identity test for increasing number of loci (PI=the probability that two individuals drawn at random from a population will have an identical genotype across multiple loci), according to Waits et al 2001. To overcome PI bias due to higher levels of shared ancestry (Taberlet and Luikart, 1999) especially in small and isolated populations where the risk of inbreeding is higher, we calculated the equivalent probability for pairs of siblings (PIsibs, Waits et al 2001).
In order to compare the SNP resolution power between Italian populations and Scandinavian population, we processed Alpine and Apennine genotypes together with 19 complete genotypes belonging to the Scandinavian bear population, for which the markers were developed, repeating the analysis with three different sets of data: a) the total dataset of 85 autosomal SNPs, b) the subset of SNPs selected for the Alpine population, and c) the subset of SNPs selected for the Apennine population. Italian and Scandinavian genotypes were used for calculating values of population summary statistics.

Genotyping success and genotyping error analysis

We used the software GIMLET v. 1.3.3 (Valière 2002) to calculate percentage of positive amplifications and error rates. The consensus genotypes of 22 out of 23 triplicated samples were used as the reference genotypes (one sample was rejected because of low quality DNA). Allelic dropout (ADO) was counted from heterozygote loci as the proportion of alleles which did not amplify (Broquet and Petit 2004). Genotyping error rates were expressed per-genotype and per-locus as the proportion of observed number of erroneous genotypes on the number of genotypes and samples in which an error could have been observed. We also calculated and compared the percentage of missing data among consensus genotypes and replicates in order to assess if replicates were necessary to obtain sufficiently reliable and complete genotypes or not.

RESULTS

SNP and sample validation

A total of 52 out of 85 (61.1%) autosomal SNPs produced a usable result and were retained, whereas 9 SNPs did not amplify (Table 4.1, step 1), 13 SNPs were monomorphic in both the Alpine and the Apennine population (plus one on the X-chromosome) (Table 4.1, step 2) and 11 autosomal SNPs showed ambiguous clusters (Table 4.1, step 3). Analyzing the Alpine and the Apennine samples separately, we found 45 autosomal SNPs to be monomorphic only in one population, two in the Alpine population (one of which was already rejected because it showed an ambiguous cluster), and 43 in the Apennine population (four of which were already rejected because of ambiguous clusters and two of which were on X-chromosome) (Table 4.1 Step 4). When assessing the call rate for each sample, we removed four samples from the Alpine dataset and four from the Apennine dataset because their call rate was ≤ 50% (Table 4.1, step 5). Finally, we found one SNP from the Alpine population and two SNPs from the Apennine population on Y-chromosome that showed a call rate ≤70% on filtered samples, while all autosomal SNPs showed a call rate per SNP ≥70%.
After the filtering process, 51 autosomal SNPs plus 6 sex chromosome SNPs (4 on Y-chromosome and 2 on X-chromosome) were retained for the remaining 19 samples in the Alpine population, while 15 autosomal SNPs plus 4 sex chromosome SNPs (all on Y-chromosome) were retained for 18 samples in the Apennine population (Table 4.1, step 6). This filtered dataset was used for further analysis, while other SNPs and samples were discarded.

	ALPINE POPULATION				APENNINE POPULATION			
Filtering steps	**Remaining nuclear autosomal SNPs**	**Remaining SNPs on sexual chromosomes**	**Remaining SNPs on mtDNA**	**Remaining samples**	**Remaining nuclear autosomal SNPs**	**Remaining SNPs on sexual chromosomes**	**Remaining SNPs on mtDNA**	**Remaining samples**
-	**85**	**7**	**4**	**28**	**85**	**7**	**4**	**27**
1	76	7	4	23	76	7	4	22
2	63	6	4	23	63	7	4	22
3	52	6	4	23	52	7	4	22
4	51	6	4	23	15	4	4	22
5	51	6	4	19	15	4	4	18
6	**51**	**6**	**4**	**19**	**15**	**4**	**4**	**18**

TABLE 4.1 - FILTERING STEPS ARE LISTED WITH THEIR RESPECTIVE NUMBER OF SNPS AND SAMPLES KEPT FOR THE ANALYSIS

The second line, highlighted in light grey, shows total number of different types of SNPs included in the original 96x96 panel and the total number of analyzed samples. The number of samples and SNPs for each filter decreases in lines accordingly to the criteria described in the text (paragraph "SNP and sample validation") and listed in the first column. The last line summarizes the number of SNPs and samples not discarded and used for further analysis.

Species confirmation and sex determination

When we used the mitochondrial markers to confirm the target species of the filtered samples, we found both Alpine and Apennine samples to have the same haplotypes at mitochondrial markers. All 19 Alpine samples and 18 Apennine samples indeed belong to the target species.
With Alpine samples, we compared results on sex determination obtained with 4 Y-chromosome and 2 X-chromosome SNPs with results obtained amplifying the SRY and AMG genes. Regarding the Apennine samples, we compared results of 4 Y-chromosome SNPs with results of only the AMG gene. The sex determination carried out by the use of SNPs was in agreement with the results obtained through microsatellites in 13 out of 19 Alpine samples and in 16 out of 20 Apennine samples, whereas 4 Alpine and 4 Apennine samples were stated undetermined. The reason is due to the insufficient number of calls in the sex-chromosome loci.

Genetic variability of selected SNPs and individual identification

We determined the individual genotypes by using the selected subsets of SNPs and we compared values of population summary statistics obtained with the selected SNP and STRs. Mean scored values are summarized in table 4.2. MAF values were always ≥0.05 with the exception of two SNPs in the Alpine population and three in the Apennine population (table 4.3). All SNP and STR loci appeared to be in Hardy-Weinberg equilibrium when significance levels are adjusted using the Bonferroni correction.

The 51 SNPs were able to distinguish individuals of the Alpine population (PID $7{,}9x10^{-17}$, PIDsibs $5{,}5x10^{-9}$) providing better resolution power to that derived from 15 STRs genotyped in the same samples (PID $3{,}8x10^{-13}$, PIDsibs $6{,}4x10^{-6}$) (Figure 4.2a). In contrast, 15 SNPs were not enough to distinguish individuals of Apennine population (PID $1{,}5x10^{-4}$, PIDsibs $1{,}1x10^{-2}$) and were less powerful than 11 STRs genotyped in the same samples (PID $3{,}7x10^{-5}$, PIDsibs $5{,}9x10^{-3}$) (Figure 4.2b). Our results indicate that, in the Alpine population, ~37 SNPs provide a resolution in identifying individuals comparable to that provided by 15 STRs (PIDsibs with 37 SNPs: $7{,}4x10^{-6}$; PIDsibs with 15 STRs $6{,}4x10^{-6}$). We obtained extremely low PID and PIDsibs values with the total set of 85 SNPs in the Scandinavian population (PID $9{,}7x10^{-33}$, PIDsibs $2{,}3x10^{-17}$), and low values when using 51 and 15 SNPs, selected to be suitable for the Alpine and the Apennine Italian populations (51 SNPs: PID $1{,}2x10^{-19}$, PIDsibs $1{,}4x10^{-10}$) (15 SNPs: PID $1{,}5x10^{-6}$, PIDsibs $9{,}0x10^{-4}$). PID and PIDsibs values of Scandinavian samples are plotted together with values obtained with Alpine samples (Figure 4.3a) and Apennine samples (Figure 4.3b) for comparisons.

Markers and		**Na**	**Ne**	**I**	**Ho**	**He**	**UHe**	**F**	**MAF**
85 SNPs	Mean	**2.00**	**1.80**	**0.62**	**0.45**	**0.43**	**0.44**	**-0.04**	**0.35**
Scandinavian	SE		0.02	0.01	0.02	0.01	0.01	0.03	0.11
51 SNPs-	Mean	**2.00/2.00**	**1.79/1.61**	**0.61/0.53**	**0.43/0.39**	**0.43/0.35**	**0.44/0.36**	**0.00/-0.08**	**0.35/0.27**
Scandinavian	SE		0.03/0.04	0.02/0.02	0.02/0.02	0.01/0.01	0.01/0.01	0.04/0.03	0.12/0.13
15 SNPs	Mean	**2.00/2.00**	**1.84/1.48**	**0.64/0.44**	**0.52/0.28**	**0.45/0.28**	**0.46/0.29**	**-0.15/-0.01**	**0.37/0.21**
Scandinavian	SE		0.05/0.08	0.02/0.05	0.04/0.04	0.02/0.04	0.02/0.04	0.08/0.08	0.10/0.15
15 STRs	Mean	**4.67**	**3.38**	**1.28**	**0.69**	**0.67**	**0.69**	**-0.03**	-
Alpine	SE	0.25	0.24	0.08	0.05	0.04	0.04	0.03	-
11 STRs	Mean	**2.18**	**1.89**	**0.66**	**0.47**	**0.44**	**0.45**	**-0.03**	-
Apennine	SE	0.12	0.14	0.07	0.07	0.05	0.05	0.08	-

TABLE 4.2 - POPULATION GENETICS SUMMARY STATISTICS

Number of alleles (Na), effective number of alleles (Ne), Shannon's information index (I), observed (Ho) and expected (He) heterozygosity, unbiased expected heterozygosity (UHe) and fixation index (F) for Alpine, Apennine and Scandinavian populations. The first line shows values obtained with Scandinavian genotypes and the total dataset of 85 autosomal SNPs available in the 96x96 panel. Second and third lines compare values of selected subsets of SNPs (51 for the Alpine population and 15 for the Apennine population) when used with Scandinavian (on the left) or Italian genotypes (on the right). Fourth and fifth lines show values reported for Alpine and Apennine genotypes with 15 and 11 STRs respectively. Mean values are in bold, while the corresponding standard error values (SE) are below. MAF values are calculated only for SNP markers.

SNP code	Base pairs	N of calls	Freq allele 1	Freq allele 2	MAF	Ne	I	Ho	He	UHe	F
snp_104	A/C	18	0,22	0,78	0,22	1,53	0,53	0,33	0,35	0,36	0,04
snp_105	C/T	19	0,47	0,53	0,47	1,99	0,69	0,42	0,50	0,51	0,16
snp_112	G/T	19	0,50	0,50	0,50	2,00	0,69	0,79	0,50	0,51	-0,58
snp_114	G/T	19	0,79	0,21	0,21	1,50	0,51	0,42	0,33	0,34	-0,27
snp_116	C/T	19	0,74	0,26	0,26	1,63	0,58	0,21	0,39	0,40	0,46
snp_118	A/G	18	0,39	0,61	0,39	1,91	0,67	0,56	0,48	0,49	-0,17
snp_119	A/G	19	0,18	0,82	0,18	1,43	0,48	0,37	0,30	0,31	-0,23
snp_120	G/T	19	0,61	0,39	0,39	1,92	0,67	0,68	0,48	0,49	-0,43
snp_128	A/G	18	0,75	0,25	0,25	1,60	0,56	0,50	0,38	0,39	-0,33
snp_129	C/T	17	0,41	0,59	0,41	1,94	0,68	0,82	0,48	0,50	-0,70
snp_131	G/T	18	0,11	0,89	0,11	1,25	0,35	0,22	0,20	0,20	-0,13
snp_134	C/T	19	0,87	0,13	0,13	1,30	0,39	0,26	0,23	0,23	-0,15
snp_136	A/C	19	0,11	0,89	0,11	1,23	0,34	0,21	0,19	0,19	-0,12
snp_141	A/G	19	0,89	0,11	0,11	1,23	0,34	0,21	0,19	0,19	-0,12
snp_162	A/G	17	0,76	0,24	0,24	1,56	0,55	0,47	0,36	0,37	-0,31
snp_164	A/T	19	0,08	0,92	0,08	1,17	0,28	0,16	0,15	0,15	-0,09
snp_168	C/T	19	0,82	0,18	0,18	1,43	0,48	0,37	0,30	0,31	-0,23
snp_169	A/G	19	0,89	0,11	0,11	1,23	0,34	0,21	0,19	0,19	-0,12
snp_170	C/T	18	0,67	0,33	0,33	1,80	0,64	0,33	0,44	0,46	0,25
snp_172	C/T	19	0,24	0,76	0,24	1,57	0,55	0,47	0,36	0,37	-0,31
snp_176	C/T	19	0,74	0,26	0,26	1,63	0,58	0,42	0,39	0,40	-0,09
snp_179	C/G	19	0,53	0,47	0,47	1,99	0,69	0,63	0,50	0,51	-0,27
snp_180	C/T	19	0,84	0,16	0,16	1,36	0,44	0,21	0,27	0,27	0,21
snp_183	A/T	19	0,97	0,03	0,03	1,05	0,12	0,05	0,05	0,05	-0,03
snp_186	C/T	19	0,45	0,55	0,45	1,98	0,69	0,47	0,49	0,51	0,04
snp_191	A/C	19	0,26	0,74	0,26	1,63	0,58	0,42	0,39	0,40	-0,09
snp_195	A/T	18	0,61	0,39	0,39	1,91	0,67	0,44	0,48	0,49	0,06
snp_199	C/T	19	0,16	0,84	0,16	1,36	0,44	0,11	0,27	0,27	0,60
snp_200	A/G	17	0,15	0,85	0,15	1,33	0,42	0,29	0,25	0,26	-0,17
snp_201	A/G	19	0,37	0,63	0,37	1,87	0,66	0,53	0,47	0,48	-0,13
snp_202	A/G	17	0,47	0,53	0,47	1,99	0,69	0,47	0,50	0,51	0,06
snp_203	A/G	19	0,18	0,82	0,18	1,43	0,48	0,37	0,30	0,31	-0,23
snp_204	A/C	18	0,83	0,17	0,17	1,38	0,45	0,33	0,28	0,29	-0,20
snp_205	C/T	18	0,83	0,17	0,17	1,38	0,45	0,22	0,28	0,29	0,20
snp_206	G/T	19	0,84	0,16	0,16	1,36	0,44	0,32	0,27	0,27	-0,19
snp_209	C/T	18	0,36	0,64	0,36	1,86	0,65	0,72	0,46	0,47	-0,57
snp_211	A/G	17	0,38	0,62	0,38	1,90	0,67	0,29	0,47	0,49	0,38
snp_212	C/T	19	0,53	0,47	0,47	1,99	0,69	0,42	0,50	0,51	0,16
snp_213	C/T	19	0,50	0,50	0,50	2,00	0,69	0,58	0,50	0,51	-0,16
snp_214	A/G	17	0,24	0,76	0,24	1,56	0,55	0,47	0,36	0,37	-0,31
snp_217	C/T	18	0,83	0,17	0,17	1,38	0,45	0,33	0,28	0,29	-0,20
snp_218	C/T	19	0,32	0,68	0,32	1,76	0,62	0,53	0,43	0,44	-0,22
snp_220	A/C	18	0,50	0,50	0,50	2,00	0,69	0,44	0,50	0,51	0,11
snp_221	A/G	18	0,08	0,92	0,08	1,18	0,29	0,17	0,15	0,16	-0,09
snp_223	A/G	19	0,55	0,45	0,45	1,98	0,69	0,37	0,49	0,51	0,25
snp_225	C/T	19	0,05	0,95	0,05	1,11	0,21	0,11	0,10	0,10	-0,06
snp_228	A/C	17	0,44	0,56	0,44	1,97	0,69	0,41	0,49	0,51	0,16
snp_230	C/T	17	0,82	0,18	0,18	1,41	0,47	0,35	0,29	0,30	-0,21
snp_234	C/T	18	0,36	0,64	0,36	1,86	0,65	0,61	0,46	0,47	-0,32
snp_239	C/T	18	0,28	0,72	0,28	1,67	0,59	0,33	0,40	0,41	0,17
snp_241	A/G	19	0,42	0,58	0,42	1,95	0,68	0,53	0,49	0,50	-0,08

TABLE 4.3 - ALPINE POPULATION – GENETIC VARIABILITY OF SELECTED SNPS

SNP codes, base pairs, number of calls, number of allele frequencies (freq allele 1 and freq allele 2), MAF values, effective number of alleles (Ne), Shannon's information index (I), observed (Ho) and expected (He) heterozygosity, unbiased expected heterozygosity (UHe) and fixation index (F) for 23 brown bear individuals from the Alps.

SNP code	Alleles	N of calls	Freq allele 1	Freq allele 2	MAF	Ne	I	Ho	He	UHe	F
snp_104	A/C	18	0,03	0,97	0,03	1,06	0,13	0,06	0,05	0,06	-0,03
snp_112	G/T	14	0,39	0,61	0,39	1,91	0,67	0,64	0,48	0,49	-0,35
snp_119	A/G	17	0,12	0,88	0,12	1,26	0,36	0,24	0,21	0,21	-0,13
snp_120	G/T	18	0,42	0,58	0,42	1,95	0,68	0,28	0,49	0,50	0,43
snp_128	A/G	18	0,03	0,97	0,03	1,06	0,13	0,06	0,05	0,06	-0,03
snp_131	G/T	16	0,06	0,94	0,06	1,13	0,23	0,13	0,12	0,12	-0,07
snp_162	A/G	17	0,41	0,59	0,41	1,94	0,68	0,35	0,48	0,50	0,27
snp_166	C/T	16	0,13	0,88	0,13	1,28	0,38	0,25	0,22	0,23	-0,14
snp_170	C/T	15	0,83	0,17	0,17	1,38	0,45	0,33	0,28	0,29	-0,20
snp_191	A/C	17	0,85	0,15	0,15	1,33	0,42	0,18	0,25	0,26	0,30
snp_199	C/T	17	0,62	0,38	0,38	1,90	0,67	0,18	0,47	0,49	0,63
snp_206	G/T	15	0,30	0,70	0,30	1,72	0,61	0,47	0,42	0,43	-0,11
snp_209	C/T	18	0,72	0,28	0,28	1,67	0,59	0,56	0,40	0,41	-0,38
snp_223	A/G	16	0,97	0,03	0,03	1,06	0,14	0,06	0,06	0,06	-0,03
snp_228	A/C	15	0,23	0,77	0,23	1,56	0,54	0,47	0,36	0,37	-0,30

TABLE 4.4 - APENNINE POPULATION – GENETIC VARIABILITY OF SELECTED SNPS

SNP codes, base pairs, number of calls, number of allele frequencies (freq allele 1 and freq allele 2), MAF values, effective number of alleles (Ne), Shannon's information index (I), observed (Ho) and expected (He) heterozygosity, unbiased expected heterozygosity (UHe) and fixation index (F) for 22 brown bear individuals from the Apennines.

Fig.4.2 (a) - Alpine population

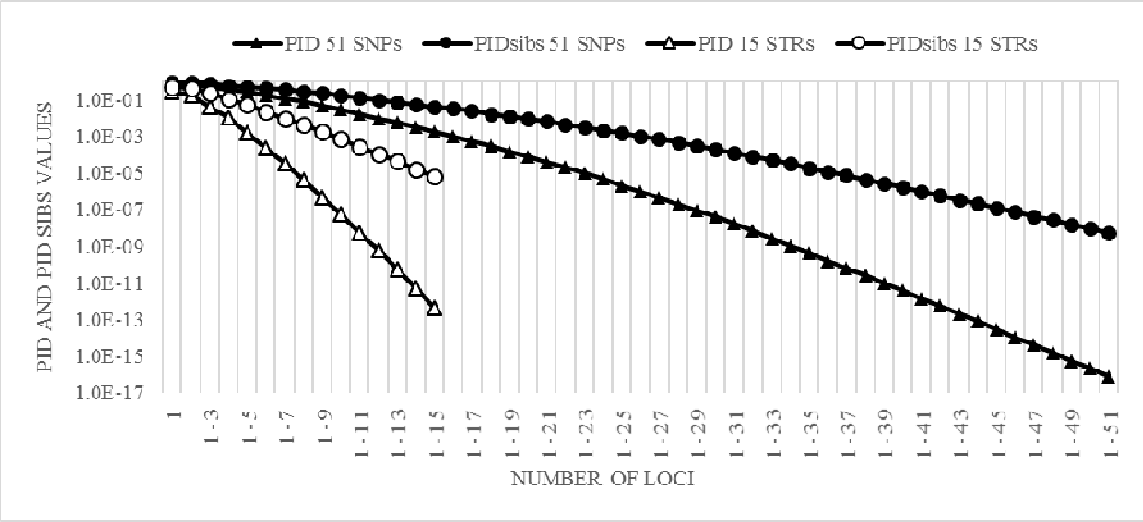
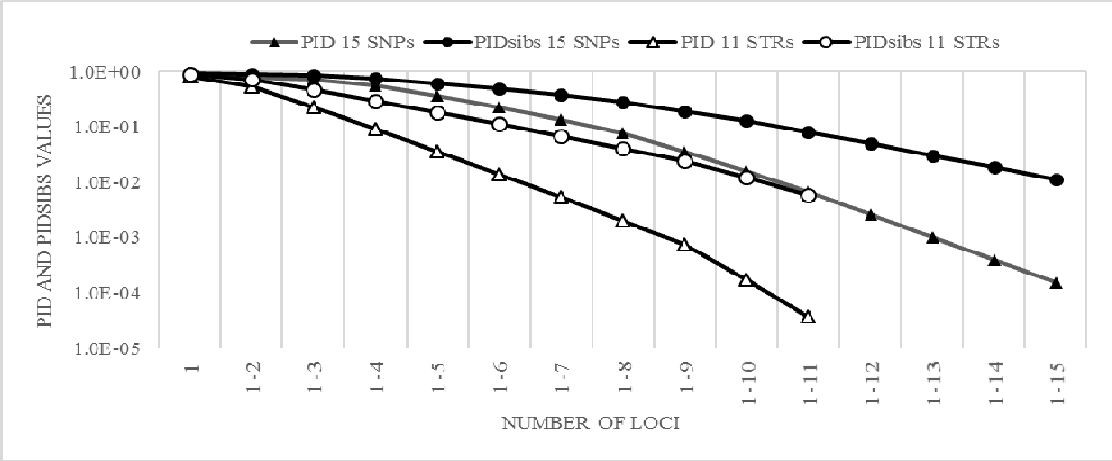


Fig. 4.2 (b) - Apennine population



FIGS. 4.2(A)-4.2(B) - PID AND PIDSIBS VALUES FOR INCREASING LOCUS COMBINATION IN THE ALPINE POPULATION (FIGURE 4.2A) AND APENNINE POPULATION (FIGURE 4.2B)

Lines with dark symbols represent PID and PIDsibs values in SNPs, while lines with white symbols represents PID and PIDsibs values in STRs. Triangles are used for PID values and dots for PIDsibs values.

Fig 4.3 (a) - Alpine and Scandinavian population

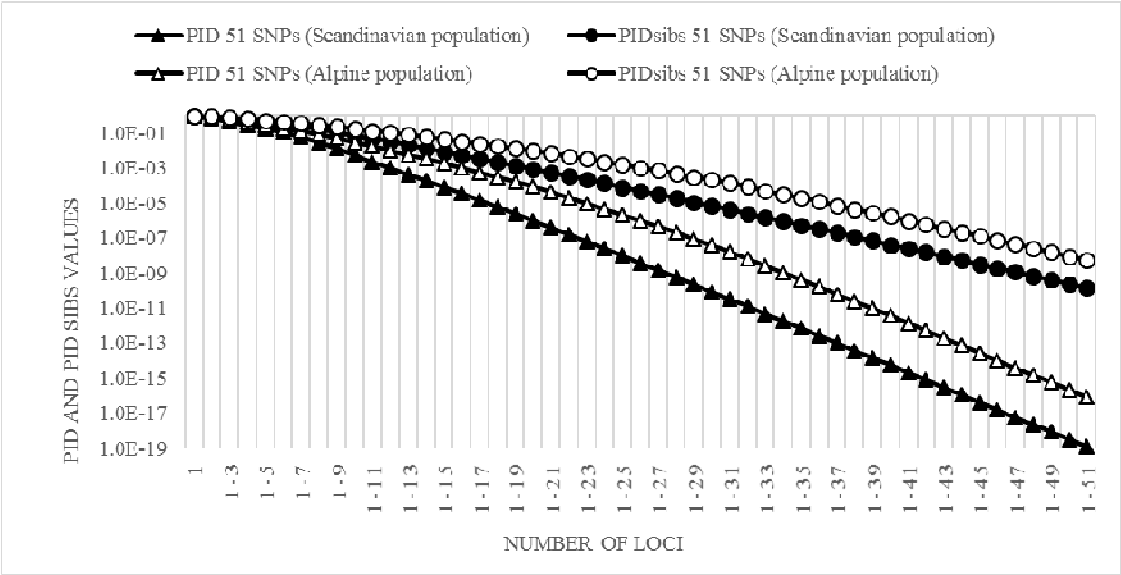
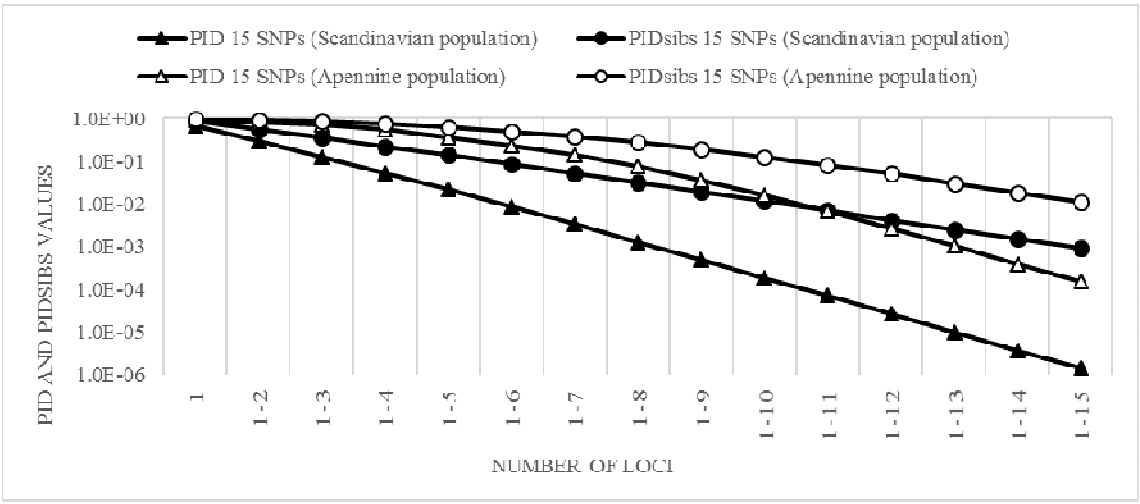


Fig 4.3 (b) - Apennine and Scandinavian population



FIGS 4.3(A)-4.3 (B) - PID AND PIDSIBS VALUES FOR INCREASING LOCUS COMBINATION IN THE SCANDINAVIAN POPULATION TOGETHER WITH ALPINE POPULATION (FIGURE 4.3A) AND APENNINE POPULATION (FIGURE 4.3B)
Lines with dark symbols represent PID and PIDsibs values in SNPs, while lines with white symbols represents PID and PIDsibs values in STRs. Triangles are used for PID values and dots for PIDsibs values.

SNP Genotyping success and errors

The SNP genotyping success was high in both populations: 14 Alpine triplicated samples showed a mean amplification rate of 90% across loci (SD=0.07) and samples (SD=0.12), while the 8 Apennine samples showed 84% of positive amplifications per locus (SD= 0.08) and per sample (SD=0.21).
Missing data calculated on consensus genotypes of retained good quality samples were scarce (3.0% in the Alpine population and 8.5% in the Apennine population). Values for missing data, calculated on replicates, are not much different in the Alpine population (2.5%), while the value is slightly worse (5.8%) in the Apennine population.
Genotyping errors occurred in the form of ADO in 1.1% (SD=0.030) of loci and 1.4% (SD=0.022) of samples and in 5% (SD=0.140) of loci and 1.8% (SD=0.051) of samples in the Alpine and Apennine populations respectively.

DISCUSSION

SNPs are often referred to as the "new" genetic marker of choice (Brumfield et al 2003) thanks to their ability to overcome STRs weaknesses with non-invasive DNA samples, such as high genotyping error rates and low amplification success. Moreover, SNPs have the advantage of cross-laboratory compatibility, thanks to their unequivocal bi-allelic status. Italian bear populations are expanding their distribution range to historical areas (Forconi et al 2014; Tosi et al 2015), whose jurisdiction are now in the hand of multiple management agencies, which often rely on different laboratories for the genetic analysis. For this reason, cooperation among jurisdictions and standardization of genetic markers is of urgent importance. SNPs offer particular promise in this regard. Yet, despite these advantages, a single bi-allelic SNP holds less information and has less statistical power compared to a single multiallelic STR, thus a sufficient number of informative SNPs need to be identified in order to performs well in population genetic studies (Seddon et al 2005; Hauser et al 2011). The 96 SNPs used in the panel developed by Norman et al (2013) were selected to maximize the informativeness in the Scandinavian population. Since the Italian populations have been separated from the Scandinavian population for thousands of years, the SNPs were expected to have less statistical power, or indeed completely lack variation, due to ascertainment bias (Morin et al 2004). Only a subset of the 96 SNPs selected for the Scandinavian populations was therefore expected to perform well in Italian brown bear populations. Indeed, 51 and 15 out of 85 autosomal SNPs were found to be informative for the Alpine and the Apennine populations respectively, and, as expected, PID and PIDsibs values were lower in the Scandinavian population using the same subset of SNPs (Figures 4.2a and 4.2b). Even if the information content is sufficient for the identification of individuals in the Alpine population, values of summary statistics (Table 4.2) suggest a moderate ascertainment bias. Given our results, we emphasize the importance of testing the information content when transferring SNPs across populations, since SNPs are demonstrated to be even more affected by ascertainment bias than STRs (Morin et al 2004).

In the Alpine population, the PIDsibs value shows that 51 SNPs have a higher resolution power to that provided by 15 STRs (Figure 4.2a). Low levels of PIDsibs are an advantage for the genetic monitoring of populations with presence of close relatives (Taberlet and Luikart 1999), as in the case of this population (De Barba et al 2010), since the probability of finding individuals sharing the same genotype by chance is lower. We also found that, in the Alpine population, ~37 SNPs are sufficient to provide a resolution in identifying individuals comparable to that provided by 15 STRs (Figure 4.2a). The number of SNPs required to match the resolution with microsatellites is theoretically 2-6X (Morin et al 2004), but several studies demonstrated that <1-3X as many SNPs can perform equally well (e.g. Ryynanen et al 2007; Coates et al 2009). Our data provide additional evidence, since we obtained the same resolution power with 2.5 times the number of SNPs. Moreover, our data show that 24 SNPs are sufficient to obtain an appropriate PID (Figure 4.2a) to reliably distinguish individuals in these small and isolated populations. In the Alpine population, we found many more informative loci (51) than those required, and thus the subset of SNPs obtained with this study is sufficient for a reliable analysis.

Conversely, in the Apennine population, the 15 reliable and informative SNPs are not sufficient for obtaining a PID equivalent to that of the 11 STRs (Figure 4.2b). The low number of informative SNPs in the Apennine population is due to the high rate of monomorphic loci (43), probably caused by the very low genetic diversity (Lorenzini et al 2004) and the high levels of homozygosity (Benazzo et al 2015) of this population. Our results are concordant with preliminary data on genome analysis of this species processed by Benazzo et al 2015, which show that Apennine bear genomes have only 1/3 of SNPs found in other European bears and that they are highly homogenous, with long stretches of homozygosity. Our study highlights the need of *de novo* SNP discovery in the Apennine population to obtain an acceptable resolution power for individual identification. However, monomorphic loci, even if not useful for *fingerprinting* analysis, can be used for other purposes, such as phylogeographic analysis, inbreeding, or population history studies.

SNPs are proving to be efficient in sex determination, since results were concordant with previous results using STRs. Many sex-chromosome SNP can be analyzed contemporaneously on a SNP chip, compared to the number of STRs normally analyzed in a routine monitoring project. If a mutation occurs at the single STR locus, errors in sex determination may happen. The same problem is less likely to cause errors in sex determination if more than one locus is used.

Missing data on good quality samples are lower than 10% as in other studies, as for example in Kraus et al 2015. Since the number of missing data calculated on consensus genotypes and replicates do not much differ, we can conclude that replicates do not significantly increase the genotyping success. We did not include the 8 discarded samples (Table 4.1, step 5) because bad quality samples complicate the visualization of clusters in the Fluidigm SNP

genotyping analysis software v3.1.2, and therefore must be manually removed from the dataset. The manual removal attributes “0 calls” to the samples, distorting the real values of missing data. We emphasize the importance of selecting only good quality sample when using non-invasive material, in order to facilitate the cluster visualization.
The presence of genotyping errors in both STRs and SNPs single PCR amplifications necessitated the use of a multiple-tube approach that includes 4 replicates of the same extract for STR markers and 2 replicates for SNP markers, according to the low rates of genotyping failure and errors that SNPs show in comparison to STRs (ADO rates for SNPs 0.011-0.050 and STRs 0.041-0.168). Even if replicates do not significantly increase the genotyping success with SNPs, ADO can appear in the final genotypes if PCR amplifications are not replicated. We suggest to include at least 2 replicates when using SNPs for individual identification, to minimize the presence of ADO in final genotypes.
The cost of a 96x96 panel, using Fluidigm system (BioMark - Fluidigm Corporation, San Francisco, USA), for the individual genotyping with two PCR replicates was around 100 euros per sample. The cost for the individual genotyping with four PCR replicates at 17 (15 plus 2 for sex-determination) and 12 (11 plus 1 for sex determination) STR loci was of the same order of magnitude. However, costs can vary in time and depend on the number of markers needed to get a good resolution, which varies according to population genetic variability.

CONCLUSIONS

We found a set of 51 effective, informative and reliable SNPs in the Alpine brown bear population, which show high genotyping success and low rates of genotyping errors when used with non-invasive samples. This panel could thus be used to efficiently study population size, reproductive success, genetic diversity, inbreeding, dispersal and other questions. Its use has many advantages compared to STRs, since SNPs are easily standardized markers and facilitate collaboration among laboratories involved in the study of the species in Europe. For the Apennine population, a *de novo* SNP discovery or an integration with different type of markers is needed before an acceptable resolution would be achieved.

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Chapter V - To what extent are SNP markers effective for parentage analysis with non-invasive samples? A pilot study for the small brown bear population in the Alps

ABSTRACT

Parentage analysis through molecular markers is of great importance in conservation biology, since contributes estimating breeding success, inbreeding level and effective population size, while pedigree reconstruction sheds light on the mating system, social and dispersal behavior of elusive species. The elusive and endangered brown bear survive in the Italian Alps thanks to the translocation of 9 individuals from Slovenia during the 2000s, followed by 15 years of genetic monitoring using 15 microsatellites (STRs). Since only a few of the founders reproduced, all present individuals are closely related. As generations progress, differences among individual multilocus genotypes decrease, and the identification of parentage relationships become more challenging. As a result, more informative markers are needed to perform parentage assignments with high probability values. We compare the effectiveness of different microsatellite and single nucleotide polymorphisms (SNP) marker sets in parentage analysis of the endangered brown bear population in the Italian Alps, using two parentage analysis packages: Colony and FRANz. The combination of 45 SNPs and 15 STRs provided no incongruent results with 15 STRs in parental assignments and the higher probability of assignments among correct trios when using FRANz. We propose an operating schema for a routine method to be applied when analyzing parental relationships using SNPs, taking into account challenges derived by the use of non-invasive samples.

INTRODUCTION

Molecular parentage analysis involves comparing genotypes of offspring to potential parents in order to identify parent-offspring relationships (Allendorf, Luikart & Aitken, 2013). Determination of parentage relationships has a prominent role for the conservation of wild populations in a variety of ways (Jones and Ardren 2003; Hauser et al. 2011) since it allows to determine breeding success, inbreeding level and effective population size (Wilson et al. 2002; Vonholdt et al. 2008; De Barba 2009; Stenglein et al. 2011). Determination of parentage relationships also contributes in answering ecological questions related to mating system, social and dispersal behavior of elusive species (Webster and Reichart 2005; Moore et al. 2014)(Sugg et al 1996, Sigg et al 2005) and can be used in reintroduction programs to optimize translocation strategies for endangered species (Labuschagne et al. 2015; Wright et al. 2015)

Molecular markers are a useful tool in establishing parentage relationships in wild populations when it is difficult to collect such information from field observations (Blouin 2003; Pemberton 2008). Microsatellites, also known as short tandem repeats (STRs), are multiallelic and highly polymorphic markers that have been routinely used for parentage analysis in wild populations using non-invasive samples in the last decade (Constable et al. 2001; Nielsen et al. 2001; Caniglia 2008; De Barba et al. 2010; Caniglia et al. 2014). However, STRs can be difficult to score accurately and are prone to genotyping errors when using non-invasive samples (Dewoody et al. 2006) possibly causing assignment failures or incorrectnesses in parentage assignments (Pompanon et al. 2005). In contrast, single nucleotide polymorphisms (SNPs) are becoming increasingly popular as the marker of choice for many conservation genetic studies, thanks to their ease of scoring, known mutational processes (Brumfield et al. 2003; Ellegren 2004), higher genotyping success, lower genotyping error rates (Morin et al. 2004; Anderson and Garza 2006), their abundance and broader genome coverage which comprises coding and non-coding regions (Brumfield et al. 2003). Moreover, SNP data do not require allele calibration among different laboratories and therefore genotypes can be easily compared (Pompanon et al. 2005; Vignal et al. 2008). This is particularly important for the monitoring of bears in the Alps since bears have a high dispersal capacity and often cross national borders. However, SNPs are biallelic thus show lower heterozygosity (limited to a maximum of 0.5) compared to STRs. Low heterozygosity is disadvantageous for parentage analysis, which requires high statistical power (Tokarska et al. 2009). Nevertheless, a high number of SNPs can be simultaneously analyzed on microfluidic arrays, compensating the low per-SNP information content.

SNPs have been proved to be effective in parentage identifications in a number of studies (Hauser et al. 2011; Fernández et al. 2013)(Kaiser et al. 2017), even for populations which face low genetic diversity (Tokarska et al. 2009; Wright et al. 2015), but empirical studies testing the effectiveness of SNP markers for parentage analysis in wild populations using non-invasive samples are still lacking. Non-invasive genetic methods offer a unique opportunity to infer parentage relationships in wild and elusive populations minimizing disturbance in their habitats (Taberlet et al. 1999), but DNA of non-invasively collected material is often degraded and quantity is usually low, thus their use presents considerable challenges (Navidi et al. 1992; Taberlet et al. 1999; Broquet and Petit 2004). However, the recently emerged microfluidigm platforms rely on the amplification of very short amplicons, thus are particularly suitable for the amplification of poor quality DNA, such that extracted from non-invasively collected material (Von Thaden et al. 2017).

The Italian Alpine bear population (*Ursus arctos arctos)* is situated in the central Italian Alps in isolation from the larger population, that once extended from the Dinaric mountains to the Alps. Recent studies approximate the presence of only 42 bears (CI=38-55)(Groff et al. 2017). Its geographic and numeric contraction occurred between the 18th and 20th century, as a consequence of human persecution and habitat loss and fragmentation. By the 1900s, only a few individuals survived (~3) (Kohn et al. 1995), thus the population was considered biologically extinct (Mustoni et al. 2003). Its presence nowadays is due to the translocation of 9 bears from Slovenia, that took place during the 2000s, which led to the increase of population and extension of its distribution (De Barba 2009; Tosi et al. 2015). A few individuals of the same subspecies (mostly males) are annually present in the eastern Italian Alps, thanks to the spontaneous recolonization from the Dinaric population. However, gene flow between the Alpine and Dinaric populations has never been observed (Krofel et al. 2010; Skrbinšek et al. 2012)

Although the brown bear is considered to be at least concern in its worldwide distribution area (McLellan et al 2017), it is critically endangered in the Italian Alps (Fusillo, Lapini & Zibordi, 2016) due to the limited numeric consistency and geographic isolation, and thus requires an active and continuous monitoring conservation policy. Parentage analysis offers the opportunity to keep track of breeding success, inbreeding level, effective population size over time and to investigate dispersal and mating behavior. All these aspects are particularly important following reintroductions to monitor the status and probability of population persistence (Sarrazin and Barbault 1996).

Fifty-one autosomal and six sexual chromosome SNP markers were previously selected for their ability in distinguish individuals and determining sex in the Alpine bear population (Giangregorio et al, submitted). Here we test the 57 SNPs for the identification of parent-offspring relationships using non-invasively collected hair samples and we compare efficiency with different SNP and SNP/STR marker sets. Therefore, we provide a feasible operating schema for parentage assignments of newly identified bears for a routine method to be applied when analyzing parental relationships using SNPs, taking into account difficulties arising from the use of non-invasive samples.

Independent robust STR-based results and field data are used to confirm or reject SNP-based parental assignments. Easy access, small distribution and intensive monitoring over 15 years allowed the identification of most of the bears. The integration of field data demonstrated that only a few newborns escaped the annual genetic monitoring program, but the undetected bears were usually genetically detected one or a few more years after their year of birth (Groff et al 2015). This population offers a great opportunity to test the suitability of SNP-based parentage analysis through non-invasive samples in a small population because a) multiple sampling of the same individuals and multiple amplifications of STR loci have been conducted, thus STR genotypes have a high degree of reliability and therefore we consider their results in parentage assignments as a reference, and b) field data derived from camera-trapping, telemetry and direct observations are at disposal to confirm genetic parentage assignments in some cases and, c) it is highly probable that all parents have been sampled.

MATERIAL AND METHODS

STR genotype-databank development

The genetic monitoring program of the Alpine bear population started in 2002 following the translocation of 9 bears belonging to the Dinaric population. Until 2016 approximately 7.000 hair and fecal samples have been non-invasively collected with different sampling method: opportunistically during field patrolling, by the use of baited hair-traps, on rub-trees and during inspections of damages to beehives and livestocks, with the aim of sampling all present bears including new born individuals. Samples were delivered to the Institute for Environmental Protection and Research (ISPRA), Italy, where they were immediately stored at -20C°.
Ten STR loci were used for individual identification and sex was determined using the Amelogenin locus (AMG) (Ennis & Gallager 1994). We implemented the protocol adding 5 STR loci to perform parentage analysis and the Sex-determining Region Y (SRY) (Fechner 1996) to confirm sex. Over 15 years, extractions were conducted using different extraction methods and kits. List of primers, amplification and sequencing protocols for individual identification and sex determination are described in details in (De Barba et al. 2010), while the implementation methods are expounded in Davoli et al 2018. All DNA samples were eluted in 200 µL of elution buffer and stored at -20°. The presence of matching genotypes within the dataset was checked using GeneAlEx 6.4 (Peakall and Smouse 2012). A genotype-databank was developed, consisting of all bears sampled in the study area between 2002 and 2014.
Several precautions were taken to obtain complete and reliable multilocus genotypes: a) a multi-tube approach (Taberlet et al. 1996) consisting of four multiplexed PCR replicates was used with the aim of reducing genotyping errors such as allele drop-outs (DO) and false alleles (FA) (Broquet and Petit 2004); b) four more PCR replicates at single loci were added in case of genotyping errors or missing data. Presence of genotypes error was checked using GIMLET (Valière 2002); c) only genotypes with a reliability R score >95% calculated with RELIOTYPE (Miller et al. 2002) were not discarded; d) multiple sampling allowed to confirm genotypes, since they were obtained multiple times through independent analysis.

Sample selection and SNP genotyping

After the STR genotyping, one sample for each bear sampled in the central Italian Alps was selected for SNP genotyping. In the sample selection process, priority was given to samples which showed highest genotyping success (mean=96%, SE=0.010), absence or the lowest rate of genotyping errors among replicates, greater number of hair follicles, and sampling recentness. Three samples belonging to bears from the Dinaric populations were also selected to test parental analysis with inclusion of non-parent genotypes.
We re-extracted the samples using the Qiagene DNeasy Blood & Tissue Kit (Qiagene inc., Hilden, Germany) following the manufacturer instructions. DNA samples were eluted in 20 µL of elution buffer. The new extraction was performed to accommodate for the SNP genotyping required DNA concentration and to standardize the extraction method.
The extracted samples were sent to the Swedish University of Agricultural Sciences (SLU), Sweden, where the SNP genotyping was performed on the Biomark platform (Fluidigm Corporation, San Francisco, USA) using a 96x96 SNP panel following the amplification procedure described in Giangregorio et al (submitted). This panel, comprising 85 autosomal SNPs, 7 sex chromosome markers, and 4 mtDNA markers, was developed for population genetics studies of the Scandinavian brown bear population (Norman et al. 2013), but a previous study demonstrated the effectiveness of 51 SNPs in individual identification and 6 in sex determination of the Alpine brown bear population (Giangregorio et al submitted). The genotyping clusters were visualized in the Fluidigm SNP Genotyping analysis software v.3.1.2 in two independent combined analysis. We underline that, since this was the first data on the Alpine population, no positive samples were included. However, we visually removed all loci with unclear cluster affiliation, maintaining an extremely conservative approach in order to avoid genotyping errors. We created consensus genotypes of replicates: all allele inconsistencies among replicates were recorded as “No call” preventing genotyping errors. Individuals were identified on the basis of multilocus genotypes using autosomal SNPs while sex was determined using sex chromosome markers included in the plate, as described in Giangregorio et al (submitted). Percentage of positive amplifications and error rates were calculated among replicated samples with GIMLET v. 1.3.3 (Valière 2002), following procedures described in Giangregorio et al (submitted).

STR and SNP marker characteristics

We calculated the percentage of call rates among SNPs. Four classes of samples based on number of call rates (<70%, >=70%, >=80% and >=90%) were created. The number of family trios present in each class was used to set the call rate threshold beyond which discard samples.
Loci were tested for deviations from Hardy-Weinberg equilibrium (HWE) adjusting significance levels for multiple comparisons using the Bonferroni correction (Pemberton 2008, Cooper 1968).
We used GeneAlEx 6.4 (Peakall and Smouse 2012) to estimate a series of information content statistics: we calculated allele frequencies and genetic variability for the different marker sets among all filtered samples as number of alleles (Na) and effective number of alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He) and Shannon's information index (I). We estimated the ability of the different sets of markers in distinguishing individuals through the probability of identity test (PID), according to Waits et al. 2001. To overcome the bias caused by the presence of closely related individuals in the population, we also calculated the equivalent probability for pairs of siblings (PIDsibs), which are more likely to share identical genotype by chance (Waits et al. 2001). To estimate the combined potential of loci for paternity allocation with the different marker sets, we calculated the probability of excluding a false parent when no information exists of any true parent (PE).

Parentage analyses

Parentage relationships were evaluated using two commonly used likelihood-based parentage assignment packages: Colony v. 2.0.6.4 (Jones and Wang 2010) and FRANz v.2 (Riester et al. 2009). The parentage tests were repeated using the different marker sets and assignment results were compared. Both software packages were run using 13 distinct sample datasets, one per year of monitoring, avoiding multi-generation analyses, which are not supported by Colony. Each subset comprises bears sampled for the first time in the year of reference (for which parents need to be identified) and all putative parents. We considered as putative parents those bears that reached the reproductive age, which is 3 years for females and 4 years for males. Year of entering in the reproductive age in this population was empirically evaluated thanks to the long-term monitoring program. FRANz was also run without creating 13 years datasets, but analyzing all bears together, since this software permits multi-generation analyses. When running both software, sex information for parents, derived from AMG and SRY data, was included. All other input parameters were set as indicated in Davoli et al 2018. The number of congruent, missing and incongruent parentage assignments were calculated for all marker sets on both software packages.

RESULTS

STR and SNP genotyping and marker characteristics

The STRs genotyping, routinely used for the genetic monitoring of the population, showed the presence of 87 individuals, 40 females and 47 males in the central Italian Alps between 2002 and 2014. AMG and SRY data were always concordant in sex determination. Corresponding 87 samples were re-extracted together with the 3 samples belonging to bears from the Dinaric population, with the exception of 16 samples for which organic material was no longer available (71 samples; 81,6% of the Alpine population).
For the SNP genotyping, 5 out of 51 autosomal loci were removed, since have shown <= 70% call rate. One SNP was not in Hardy Weinberg equilibrium and was rejected for further analysis, for a total of 45 SNPs kept.
Twenty out of 74 samples showed a percentage of missing call <=70% and were rejected. Among the remaining samples, 54 had values >=70%, 40>=80% and 19>=90%. Since too many family trios would have removed rejecting samples with 80% or 90% of call rates, we decided to keep the value <=70% as a threshold. Thus 54 reliable samples, 5 of which are founders, were kept to be processed for further analyses on marker characteristics and parentage analysis.
Since the 45 SNPs were selected for a different population, not all loci showed the three genotypic representatives (AT/TT/AA), with the exception of the 27 SNPs, which are therefore the most reliable loci among 45. We therefore created 6 genotype-datasets for the selected genotypes using different set of markers: 10 STRs loci (=set a), 15 STRs (=set b), 27 SNPs (=set c), 45 SNPs (= set d), combinations of 15 STRs and 27 SNPs (=set e) and 15 STRs and 45 SNPs (= set f).
Summary statistics for all marker combinations are shown in details in table 5.3 while average statistics data of all marker sets are summarized in Table 5.2. PID and PIDsibs values are plotted in Fig. 5.1.
The genotyping of 45 autosomal SNPs correctly identified 54 bears, and sex determination based on 6 chromosome SNPs confirmed sex STR-based results in 42 out of 54 cases (Table 5.1). The remaining 12 cases did not show incongruent results but missing data at sex-loci prevented the sex determination.
The replicated samples showed 87% of positive PCR amplifications among loci and 85% of samples. Genotyping errors occurred in the form of ADO in 2.6% (SD=0.02) of loci and 2.5% (SD=0.03) of samples.

Bear ID	Sex	Year of birth	N replicates	SNP Loci Typed (Out of 45)	Genotyping success
Gasper	**M**		1	42	0.93
Maja	**F**		2	42	0.93
Daniza	**F**	**Founders**	4	45	1
Joze	**M**		2	37	0.82
Jurka	**F**		2	43	0.96
KJ1	**F**	2002	2	43	0.96
KJ2	**F**	2002	4	45	1
MJ2	**F**	2003	2	42	0.93
DJ1	**F**	2004	2	38	0.84
DJ3	**F**	2004	4	45	1
JJ2	**M**	2004	2	45	1
MJ4	**M**	2005	4	44	0.98
JJ4	**F**	2006	1	44	0.98
JJ5	**M**	2006	4	45	1
KJ2G1	**F**	2006	2	44	0.98
KJ2G2	**M**	2006	4	45	1
MJ5	**M**	2005	2	45	1
DG3	**F**	2006	2	36	0.8
KJ1G1	**F**	2006	2	45	1
BJ1	**F**	2005	4	45	1
MJ2G1	**M**	2006	1	42	0.93
F1	**F**	2008	1	45	1
F3	**F**	2008	1	45	1
F2	**F**	2008	4	45	1
M2	**M**	2008	4	45	1
M3	**M**	2008	2	44	0.98
M4	**M**	2008	2	45	1

Bear ID	Sex	Year of birth	N replicates	SNP Loci Typed (Out of 45)	Genotyping success
F4	**F**	2008	4	45	1
M6	**M**	2007	4	45	1
M7	**M**	2009	1	39	0.87
F9	**F**	2010	1	42	0.93
M9	**M**	2010	1	44	0.98
F10	**F**	2010	1	45	1
M11	**M**	2011	1	44	0.98
M12	**M**	2010	1	44	0.98
M13	**M**	2010	1	45	1
M14	**M**	2010	1	38	0.84
M15	**M**	2012	1	39	0.87
F12	**F**	2011	1	44	0.98
F13	**F**	2012	1	42	0.93
M18	**M**	2012	1	44	0.98
M19	**M**	2012	2	43	0.96
M21	**M**	2012	1	40	0.89
F14	**F**	2012	1	43	0.96
F15	**F**	2013	2	45	1
F18	**F**	2013	2	40	0.89
M25	**M**	2012	1	43	0.96
M26	**M**	2013	1	44	0.98
F19	**F**	2014	1	42	0.93
F20	**F**	2014	1	44	0.98
F21	**F**	2014	1	45	1
Gen03	**M**		4	44	0.98
Gen04	**M**	**Dinaric population**	1	40	0.89
Gen14	**M**		4	44	0.98

TABLE 5.1 - SAMPLE INFORMATION

For each of the 54 individuals of Alpine brown bears Sex (M=male, F=female), year of birth, N of replicates, number of SNP loci typed in the consensus genotype, and genotyping success are provided. Genotyping success is calculated as the proportion of loci typed among 45 autosomal SNPs.

Marker sets	Mean samples typed	N alleles	Na	Ne	I	Ho	He	PE
10 STRs	51.00 (0.000)	50	5.00 (0.149)	3.71 (0.129)	1.41 (0.024)	0.81 (0.012)	0.72 (0.009)	$9.9998x10^{-01}$
27 SNPs	49.25 (0.368)	54	2.00 (0.000)	1.75 (0.048)	0.60 (0.022)	0.43 (0.026)	0.41 (0.019)	$9.9958x10^{-01}$
15 STRs	51.00 (0.000)	69	4.6 (0.321)	3.27 (0.227)	1.25 (0.086)	0.73 (0.040)	0.65 (0.040)	$9.9999x10^{-01}$
45 SNPs	48.86 (0.374)	90	2.00 (0.000)	1.59 (0.044)	0.52 (0.022)	0.38 (0.022)	0.34 (0.019)	$9.9998x10^{-01}$
27SNPs+15STRs	49.88 (0.269)	123	2.92 (0.224)	2.29 (0.142)	0.83 (0.059)	0.54 (0.032)	0.50 (0.026)	$9.9999x10^{-01}$
45SNPs+15STRs	49.40 (0.305)	159	2.65 (0.166)	2.01 (0.114)	0.70 (0.049)	0.47 (0.028)	0.42 (0.024)	$9.9999x10^{-01}$

TABLE 5.2 - MARKER SUMMARY STATISTICS

Mean number of samples typed, total number of alleles (N alleles), mean number of alleles per locus (Na), mean effective number of alleles (Ne), Shannon's information index (I), observed (Ho) and expected (He) heterozygosity, in a total of 51 brown bear samples from central Italian Alps. Standard error values (SE) are below.

Locus	N samples typed	Na	Ne	I	Ho	He	DF	ChiSq	Prob	Significance
cxx20	51	4	3.73	1.35	0.84	0.73	6	6.769	0.34	ns
G10M	51	5	3.5	1.36	0.76	0.71	10	12.79	0.24	ns
G10P	51	5	4.52	1.55	0.86	0.78	10	16.27	0.09	ns
G10X	51	5	3.25	1.33	0.8	0.69	10	14.76	0.14	ns
G1D	51	6	3.38	1.41	0.78	0.7	15	11.8	0.69	ns
Mu11	51	5	3.93	1.47	0.82	0.75	10	18.65	0.04	ns
Mu15	51	5	3.92	1.45	0.88	0.75	10	9.397	0.49	ns
Mu23	51	5	3.38	1.3	0.82	0.7	10	16.15	0.1	ns
Mu50	51	5	4.15	1.48	0.82	0.76	10	7.158	0.71	ns
Mu59	51	5	3.43	1.39	0.76	0.71	10	15.35	0.12	ns
G10C	51	4	2.24	0.94	0.59	0.55	6	8.019	0.24	ns
G10H	51	2	1.19	0.3	0.18	0.16	1	0.478	0.49	ns
G10L	51	3	2.43	0.99	0.75	0.59	3	5.016	0.17	ns
Mu09	51	7	3.75	1.54	0.8	0.73	21	12.43	0.93	ns
Mu10	51	3	2.27	0.95	0.59	0.56	3	3.383	0.34	ns
snp_104	45	2	1.5	0.52	0.42	0.33	1	3.223	0.07	ns
snp_105	50	2	1.91	0.67	0.46	0.48	1	0.055	0.81	ns
snp_114	45	2	1.7	0.6	0.58	0.41	1	7.427	0.01	ns
snp_116	50	2	1.75	0.62	0.42	0.43	1	0.017	0.9	ns
snp_118	48	2	1.95	0.68	0.63	0.49	1	3.918	0.05	ns
snp_119	48	2	1.16	0.26	0.15	0.14	1	0.297	0.59	ns
snp_120	51	2	1.99	0.69	0.59	0.5	1	1.663	0.2	ns
snp_128	50	2	1.52	0.53	0.44	0.34	1	3.978	0.05	ns
snp_129	45	2	1.64	0.58	0.53	0.39	1	5.95	0.01	ns
snp_131	51	2	1.31	0.4	0.27	0.24	1	1.291	0.26	ns
snp_134	51	2	1.17	0.27	0.16	0.14	1	0.369	0.54	ns
snp_136	51	2	1.21	0.32	0.2	0.18	1	0.603	0.44	ns
snp_141	50	2	1.24	0.35	0.22	0.2	1	0.764	0.38	ns
snp_162	39	2	1.62	0.57	0.51	0.38	1	4.637	0.03	ns
snp_164	49	2	1.18	0.28	0.16	0.15	1	0.387	0.53	ns
snp_168	44	2	1.2	0.3	0.18	0.17	1	0.44	0.51	ns
snp_170	45	2	1.75	0.62	0.36	0.43	1	1.308	0.25	ns
snp_176	51	2	1.71	0.61	0.39	0.42	1	0.157	0.69	ns
snp_179	47	2	1.93	0.67	0.55	0.48	1	1.036	0.31	ns
snp_180	51	2	1.12	0.22	0.12	0.11	1	0.199	0.66	ns
snp_183	49	2	1.32	0.41	0.29	0.24	1	1.361	0.24	ns
snp_186	50	2	1.94	0.68	0.42	0.48	1	0.87	0.35	ns
snp_191	51	2	1.78	0.63	0.49	0.44	1	0.733	0.39	ns
snp_199	50	2	1.37	0.44	0.24	0.27	1	0.574	0.45	ns
snp_200	49	2	1.38	0.45	0.33	0.27	1	1.866	0.17	ns
snp_201	50	2	1.92	0.67	0.4	0.48	1	1.389	0.24	ns
snp_202	48	2	2	0.69	0.6	0.5	1	2.094	0.15	ns
snp_203	46	2	1.57	0.55	0.35	0.36	1	0.09	0.76	ns
snp_204	49	2	1.38	0.45	0.33	0.27	1	1.866	0.17	ns
snp_205	51	2	1.31	0.4	0.27	0.24	1	1.291	0.26	ns
snp_206	51	2	1.29	0.38	0.22	0.22	1	0.047	0.83	ns
snp_209	49	2	1.96	0.68	0.53	0.49	1	0.34	0.56	ns
snp_211	51	2	1.98	0.69	0.51	0.5	1	0.044	0.83	ns
snp_213	49	2	2	0.69	0.57	0.5	1	1.007	0.32	ns
snp_217	49	2	1.35	0.43	0.31	0.26	1	1.6	0.21	ns
snp_218	49	2	1.66	0.59	0.55	0.4	1	7.086	0.01	ns
snp_220	51	2	1.99	0.69	0.65	0.5	1	4.655	0.03	ns
snp_221	51	2	1.19	0.3	0.18	0.16	1	0.478	0.49	ns
snp_223	49	2	2	0.69	0.43	0.5	1	1	0.32	ns

snp_225	51	2	1.29	0.38	0.25	0.22	1	1.088	0.3	ns
snp_228	48	2	1.75	0.62	0.33	0.43	1	2.414	0.12	ns
snp_230	48	2	1.49	0.51	0.42	0.33	1	3.324	0.07	ns
snp_234	47	2	1.72	0.61	0.51	0.42	1	2.291	0.13	ns
snp_239	51	2	1.69	0.6	0.29	0.41	1	3.921	0.05	ns
snp_241	51	2	1.84	0.65	0.39	0.46	1	1.02	0.31	ns

TABLE 5.3 - MARKER SUMMARY STATISTICS BY LOCUS

Number of samples typed, mean number of alleles per locus (Na), mean effective number of alleles (Ne), Shannon's information index (I), observed (Ho) and expected (He) heterozygosity, in a total of 51 brown bear samples from central Italian Alps. Results for deviations from Hardy-Weinberg equilibrium (HWE) are reported for each locus. The significance levels are calculated for multiple comparisons using the Bonferroni correction.

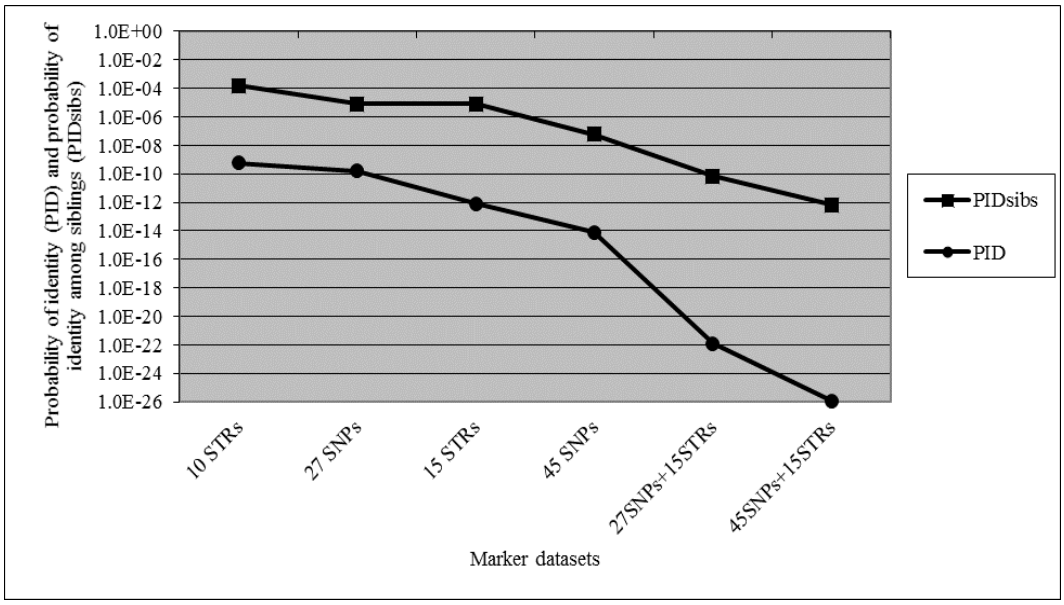

FIG 5.1 - PID AND PIDSIBS VALUES FOR INCREASING LOCUS COMBINATION IN THE BROWN BEAR ALPINE POPULATION

Values are calculated for the six marker sets. Dots are used for PIDsibs values and triangles for PID values.

Parentage analyses

Following the filtering process, 43 parental trios were still present among 51 samples. A proportion of parental assignments was confirmed by field observations thanks to intensive genetic monitoring over 15 years. Parentage assignments derived from 15 STRs genotypes and field data are listed in Table 2. The majority of offspring originated from 2 males (Gasper:=48.8%; Joze=30.2%) and 2 females (Daniza=25.5%; KJ1=20.9%).

Proportions of congruent, incongruent and missing parentage assignments are shown in Fig 5.2. The concordance/discordance is defined considering assignments obtained with 15 STRs as a reference. When using 10 STRs (a) and FRANz, all assignments were congruent with 15 STRs (b), whether 3 trios are wrongly assigned using Colony. All other marker sets find a number of incongruent results or are unable to assign parents: 27 SNPs(c) find the highest number of incongruent assignments (c-Colony=5; c-FRANz=6), followed by 45 SNPs(d), for which only a few incongruent parents are found (d-Colony=2; d-FRANz=2). SNPs alone also do not find parents in a number of cases (c-Colony=13; c-FRANz=11; d-Colony=11; d-FRANz=13). Both combinations of 27SNPs+15STRs(e) and 45SNPs+15STRs(f) do not find incongruent assignments and a proportion of parents is not assigned (e-Colony=12; e-FRANz=9; f-Colony13; f-FRANz=10). We obtained same results when running FRANz with a unique multi-generation input file, rather than using 13 different annual datasets. We underline that in most cases (mean among marker sets: 81%), parents were not assigned or assignments were incongruent when founders (Joze, Gasper, and Daniza) are involved in the trio, especially Joze that is involved in 55.5% of missing assignments. The remaining discordances in parentage assignments could be attributable to lack of replicates among individuals of the trio, to low number of call rates (e.g. Joze 37 loci typed out of 45) or genotyping errors (see table 5.1). In fact, a few mismatches are found among congruent assignments (1 MM in 7,25 trios on average of marker sets).

Bear ID	Sex	Sire	Dam
Gasper	**M**	**Founders**	
Maia	**F**		
Daniza	**F**		
Joze	**M**		
Jurka	**F**		
KJ1	**F**	Joze	Kirka*
KJ2	**F**	Joze	Kirka*
MJ2	**F**	Joze	Maya
DJ1	**F**	Joze	Daniza
DJ3	**F**	Joze	Daniza
JJ2	**M**	Joze	Jurka
MJ4	**M**	Joze	Maya
JJ4	**F**	Joze	Jurka
JJ5	**M**	Joze	Jurka
KJ2G1	**F**	Gasper	KJ2
KJ2G2	**M**	Gasper	KJ2
MJ5	**M**	Joze	Maya
DG3	**F**	Gasper	Daniza
KJ1G1	**F**	Gasper	KJ1
BJ1	**F**	Joze	Brenta*
MJ2G1	**M**	Gasper	MJ2
F1	**F**	Gasper	Daniza
F3	**F**	Gasper	Daniza
F2	**F**	Gasper	KJ1
M2	**M**	Gasper	Daniza
M3	**M**	Joze	KJ2
M4	**M**	Joze	KJ2

Bear ID	Sex	Sire	Dam
F4	**F**	Gasper	KJ1
M6	**M**	Gasper	DJ3
M7	**M**	Gasper	DJ3
F9	**F**	Gasper	KJ1
M9	**M**	Gasper	Daniza
F10	**F**	Gasper	Daniza
M11	**M**	JJ5	DJ3
M12	**M**	Gasper	KJ2
M13	**M**	Gasper	KJ2
M14	**M**	Gasper	KJ2
M15	**M**	MJ5	Daniza
F12	**F**	JJ5	F2
F13	**F**	MJ5	Daniza
M18	**M**	Gasper	KJ1
M19	**M**	M4	KJ2
M21	**M**	M6	F3
F14	**F**	Gasper	JJ4
F15	**F**	M6	F4
F18	**F**	JJ5	F2
M25	**M**	M6	KJ2
M26	**M**	M6	F4
F19	**F**	Gasper	MJ2
F20	**F**	M2	Daniza
F21	**F**	MJ5	DG3
Gen03**	**M**	Not found	Not found
Gen04**	**M**	Not found	Not found
Gen14**	**M**	Not found	Not found

TABLE 5.2 - PARENTAGE RELATIONSHIPS IN THE BROWN BEAR POPULATION IN THE ITALIAN ALPS AMONG 51 INDIVIDUALS ANALYZED IN THIS STUDY

Results are based on 15 STR loci and multiple sampling over 15 years. Results are obtained using Colony 2.0.6.4 and FRANz v.2. Sex of each bear is indicated (F= Female; M= Male). Parents marked with an asterisk (*) cannot be identified using SNPs because SNP loci were not amplified (samples no longer available or discarded bad quality sample). Three "control samples" belonging to the Dinaric population are included in the dataset and marked with a double asterisk (**).

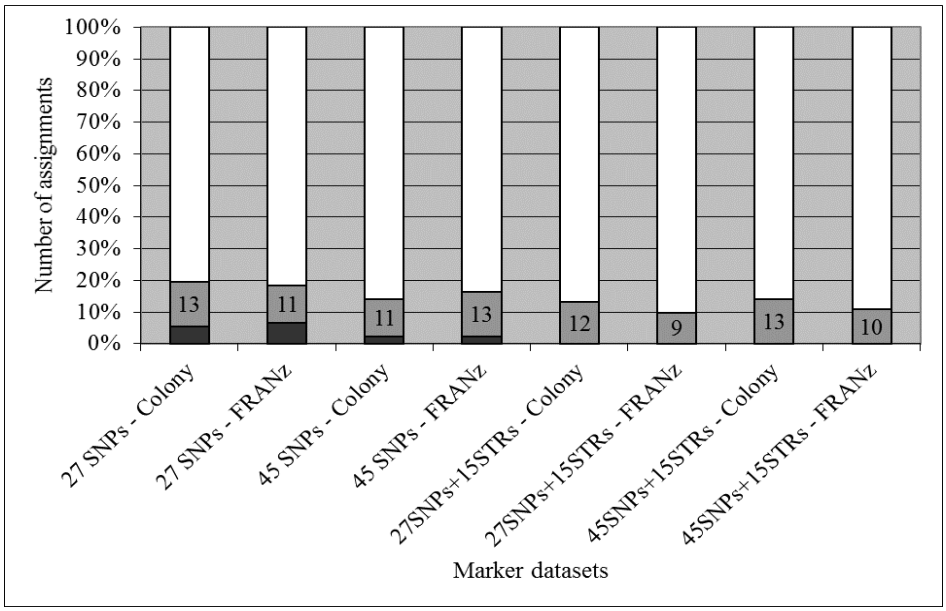

FIG. 5.2 - RATES OF PARENTAL ASSIGNMENTS

Congruent assignments are colored in white, while incongruent assignments are in dark grey. Not assigned parents are colored in light grey. Results are reported for two parentage software packages: Colony 2.0.6.4 and FRANz v.2 using 5 marker sets.

Probabilities of assignments mean values among congruent trios are lower when using SNPs alone (c=76.7%; d=89.6%), but start to increase when using STRs alone (a=93.4%; b= 98.5%). Nevertheless, best and similar results are obtained when using both combinations of SNPs and STRs (e=99.97%; f=99.99%) (Fig. 5.3)

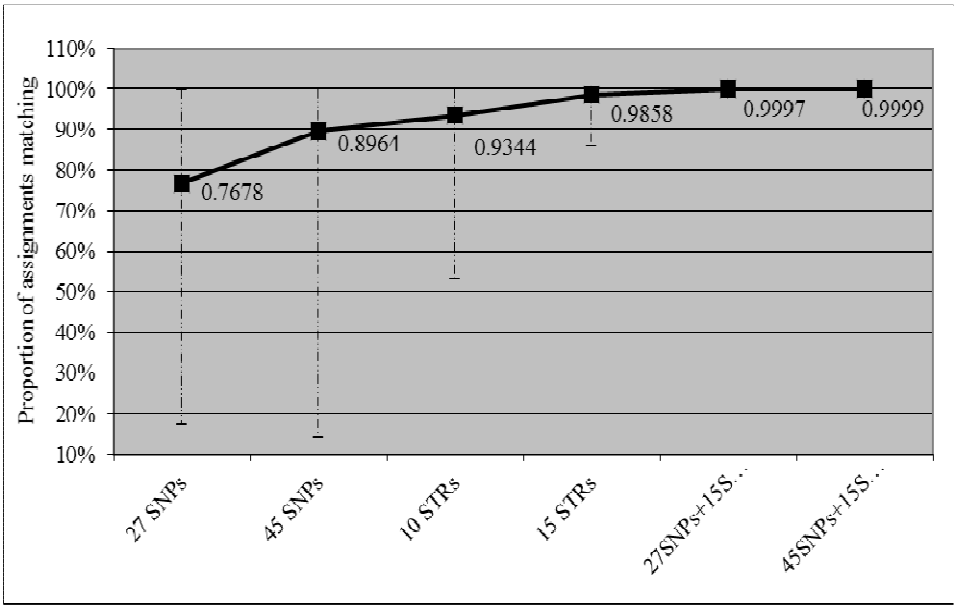

FIG. 5.3 - PROBABILITIES OF ASSIGNMENTS AMONG CONGRUENT TRIOS

Probabilities are calculated using 6 combinations of marker sets. A line with squares is used for the mean probabilities. Minimum and maximum values are marked with dashes.

DISCUSSION

In small and isolated populations, especially those descending from a limited number of founders (Tokarska et al. 2009), inbreeding events are more likely to occur, resulting in most individuals within the population to be related after a few generations (Hedrick 2000). Inbreeding events rapidly lead to reduced genetic diversity through loss and fixation of alleles, increase in homozygosity, and shifts in allele frequencies (Ralls et al. 1988)(Allendorf, Luikart and Aitken 2013). Such consequences not only have important effects on long-term survival of the population but also require the use of highly informative markers for population genetics analyses, especially for parentage assignments (Tokarska et al. 2009). As a consequence of the reduced size and isolation, the Alpine bear population is facing a slight reduction in heterozygosity (from 0.776 in 2013 to 0.656 in 2016; Groff et al 2017), despite the value remain high. Heterozygosity is expected to decrease further within next generations: empirical data show that the majority of newborns are offspring of a few individuals, inbred bears are already present (see Table 5.2: e.g. M3, M4, MJ2J1, M11, F12), and the majority of bears are closely related. We anticipate that providing demographic and genetic data (pedigree, genetic drift, inbreeding levels...) on the population is outside the scope of this study, but differential individual reproduction has consequences related to the necessity of increasing the number of markers for parentage assignments, especially in perspective of further cases of inbreeding and the increase of homozygosity. SNPs are also known to be less affected by consanguinity than STRs (Fernández et al. 2013), making this type of marker more suitable in the case of inbred populations. Moreover, it is getting more important to use new molecular markers such as SNPs, whose alleles are easily comparable between laboratories, since bears are rapidly expanding their distribution area from Italian Alps to neighboring countries and a cooperative approach is a key factor for the long-term conservation of the species in Europe.

The efficiency in finding parental assignments is slightly higher for FRANz because, unlike Colony, did not find incongruent assignments using 10 STRs, suggesting that FRANz could be more efficient when using microsatellite markers alone. Moreover, even if incongruent assignments should be considered a more serious error than missing assignments, FRANz returned a few less unassigned parents when using the two marker datasets with the greatest probabilities of assignment (the two combinations of SNP and STR loci). Most importantly, FRANz has intrinsic characteristics that make this software a better choice in the case of long-term monitoring projects: it permits the incorporation of prior info in addition to genotypes (years of birth and death), it is robust even in presence of genotyping errors, and our data confirm that FRANz is effectively able to manage multi-generation data (Riester 2009). This is advantageous with long-term monitoring programs with overlapping generations, because FRANz allows analyzing all newly sampled individuals (for which parentage relationships need to be determined) and putative parents together without creating an input file for each year.

Moreover, when having data on year of death of putative parents, only FRANz allows incorporating this information in a unique input file reducing the number of putative parents. This increases the probability of correct assignments and shortens time for the analysis.

When using both combinations of SNP and STR marker types, assignment probabilities are higher compared to 15 STRs, suggesting that a combination of SNPs and STRs is able to give more reliable data. Interesting, probability values do not much differ when using 27 or 45 SNPs, suggesting that 27 SNPs are yet sufficient to enhance data reliability.

A number of mismatches among correct trio point out the presence of a few genotyping errors among genotypes, that have not been shown among replicates (replicates not performed or genotyping error present among all replicates). These errors could be due to the absence of positive samples, which facilitate cluster assignments when using the SNP genotyping software. Moreover, multiple sampling and multiple amplification of single STR loci were performed when finding incongruent results across replicates, whether this is not possible when amplifying SNP loci for technical reasons. Moreover, not all samples were replicated when using SNPs and genotypes are not complete. Replicates have reported being essential with non-invasive samples, even when using SNPs, as well as call rate (Von Thaden et al. 2017).

Here we want to propose an operating schema for parentage analysis using SNPs and non-invasive low-quality samples collected during an annual monitoring program of endangered populations: a) amplification of STRs can be performed to identify individuals. STRs can be re-amplified at single loci when amplification fails or incongruent results are detected among 4 replicates. Sex can be determined through the amplification of sex-specific regions. The STR amplifications is used to discard low-quality samples and identify single individuals among all samples. Among multiple samples of the same individual, the sample with better results at STR amplification is chosen (lower genotyping error rate and higher positive amplifications) for SNP genotyping. b) a pilot study including parental trios,

determined by the use of STRs, should be performed. Genotypes belonging to trios which do not show incongruent parentage assignments or mismatches, that have four replicates and a high call rate, can be selected as positive samples for further analysis. The use of positive samples will help to identify cluster pattern correctly and avoid further genotyping errors. c) A SNP genetic databank, including all putative parents, can be developed. The impossibility of amplifying SNP at single loci requires the use of replicates to obtain reliable genotypes. We highlight the importance of replicating all genotypes four times in order to enhance the call rate and remove genotyping errors among putative parents. (e.g Joze in the case of Alpine brown bears). 96x96 or 48x48 plates can be used when using the Fluidigm Platform, depending on the number of individuals and SNP availability. d) SNP genotypes of the newborns can be annually analyzed parentage analysis can be performed using FRANz, combining SNP and STR genotypes.

In the case of brown bear in the Alps, we found 45 autosomal SNPs efficient for parentage analysis and more informative than 15 STRs on the basis of probabilities of assignments. Therefore, a 48x48 plate is sufficient. However, this pilot study highlighted the need for replicating all samples in the databank four times in order to increase the call rate and remove genotyping errors that caused the presence of mismatches among parentage assignments.

We highlight the ascertainment bias caused by the selection of SNPs informative for the Scandinavian bear population: the selected 45 SNPs are not the most heterozygous SNPs for this population. However, finding new markers is costly, thus testing pre-existing SNPs in preferable than finding new ones, when possible. Moreover, sharing markers allows comparing data on the population history of different populations. Eventually, a de novo SNP searching will allow replacing SNPs with lower heterozygosity values.

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FINAL CONCLUSIONS

This study showed that the use of non-invasive genetic sampling represents a powerful tool to study endangered species, confirming that non-invasive genetic sampling methods can help solving several issues that could not be addressed in any other way.

In this study the screening of a limited number of genetic markers, such as microsatellite loci, produced information reliably useful to monitor demographic parameters and geographic distribution of brown bears living in the Alps: we identified the number of reproducing individuals and their contribution to recruitment, we estimated rates of survival and described patterns of dispersal events and main causes of mortality. Population genetic parameters, such as genetic diversity, relatedness, inbreeding, and effective population size have also been measured.

Even if a positive demographic trend was evident in the reintroduced Alpine population and the geographic distribution expanded, the loss of genetic variation over generations was remarkable. As a consequence, given the genetic isolation of this population and the preferential reproduction of a few individuals, relatedness enhanced across generations and many inbreeding events were registered.

Therefore, conservation actions should be taken to reverse this negative trend and make the presence of a viable metapopulation in the Alps possible. Actions to promote the re-establishment of a geneflow with the Dinaric population should be implemented, such as further translocations of females outside the core area, the protection of ecological corridors across the Alps and the reduction of human-induced mortality.

The decrease in genetic variability negatively affects the evolutionary potential and the ability to adapt to environmental changes of populations, therefore the eventual restoration of gene flow will intensely enhance the survival potential of brown bears in the Alps.

For the future, a more intensive and homogeneous sample collection, randomized across the entire brown bear distribution in the Alps would be useful to significantly enhance the chance of sampling the majority of individuals, to better interpret the fluctuations of the studied population size and genetic diversity through time.

The decrease in genetic variation in the Alpine population not only is a threat to the survival of the population but also affected our ability of correctly identifying parent-offspring relationships because higher informativity content is requested when putative parents have similar genotypes. We tested a pre-existing SNP panel, whose SNPs were selected to be the most informative for the Scandinavian brown bear populations, in order to enhance the power in identifying individuals and determining parental relationships in the Alpine population. We tested the SNP panel on the Apennine population as well: the Apennine population is extremely endangered since it has one of the lowest rates of genetic diversity among bear populations worldwide. For this reason, It's currently impossible to effectively perform parentage analysis using microsatellites alone.

SNPs genotyping represents a near future application in non-invasive genetics as a promising and innovative faster and more reliable method to analyze low quality and quantity DNA samples like non-invasive ones. In fact, a sufficient number of polymorphic SNPs is able to ensure a reliable individual identification and parentage analysis. Moreover, SNPs are particularly suitable for the study of bears in the Alps since this kind of markers does not require allele calibrations among different laboratories. A close collaboration among all research centers involved in the study of brown bear is essential to the survival of this species, especially now that this population showed high dispersal capacity and samples of the same individuals have been collected in different countries.

We found a subset of SNPs to be informative and reliable for individual identification and sex determination in the Alpine population, while the number of polymorphic SNPs in the Apennine population was not sufficient to provide enough discrimination power. For the Apennine population, a de novo SNP discovery or an integration with different type of markers is needed before an acceptable resolution would be achieved. The complete genome of the Apennine population has recently been published, and a number of polymorphic SNPs have been identified. A filtering process to select the most informative SNPs for the purpose of identifying parent-offspring relationships is recommended.

We performed a pilot study to determine whether or not the selected subset of SNPs was efficient for the parentage analysis in the Alpine population, and we compared its reliability with that obtained using microsatellites. We found the SNPs to be efficient, but a number of incongruent or missing assignments was found, probably due to lack of replicates or low call rate in some of the genotypes. This pilot study enabled us to identify this issue, that can be easily solved providing a proper number of replicates for all genotypes, the offspring and putative parents.